Pattern Recognition Receptors
&
Dendritic Cell Function

in health and disease

ISBN: 978-90-9023306-2
Printing: Print Partners Ipskamp

The research described in this thesis was performed at the department of Tumor Immunology of the Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, The Netherlands, and was supported by a grant from the Dutch Diabetes Research Foundation (DFN 2001.00.47).

Printing of this thesis was financially supported by the following companies and foundations: Dutch Diabetes Research Foundation, Dutch Arthritis Association, ABBOTT B.V., Sanquin, tebu-bio.

Cover: Janus, an ancient Roman divinity with two faces pointing in opposite directions. God of beginnings and guardian of all entrances and gates.

Cover design and artwork: Annelou van Griensven (www.annelouvangriensven.nl)

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Een wetenschappelijke proeve op het gebied van de

Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor

aan de Radboud Universiteit Nijmegen

op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann

volgens besluit van het College van Decanen

in het openbaar te verdedigen op donderdag 11 september 2008

om 15.30 uur precies

door

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geboren op 10 april 1979

te Middelburg

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Chapter 1

General Introduction

"Science is built up of facts,

as a house is built of stones,

but an accumulation of facts

is no more a science

than a heap of stones is a house."

Henri Poincaré

The immune system

Humans are continuously exposed to and closely interact with a plethora of microorganisms. This interaction can be either beneficial or detrimental for the host. For instance, the gut is colonized with more than 10^{14} bacteria representing more than 500 different species [1]. These commensal bacteria contribute to digestion of certain polysaccharides [2, 3] and have important trophic effects on intestinal epithelial cells [3]. On the other hand, pathogenic microorganisms such as *HIV*, *Mycobacterium tuberculosis* or *Vibrio cholerae* can cause serious pathology in humans. Also commensals can pose a threat under certain conditions, like in individuals receiving chemotherapy or immuno-suppressive drugs (*e.g.* in transplantation settings) in whom opportunistic fungal infections are common [4, 5].

Innate versus adaptive immunity

Our immune system represents a defense system that is of crucial importance to prevent pathogens of establishing a potentially life-threatening infection. The human immune system is separated in two distinct but intertwined components: innate and adaptive immunity. Innate immunity consists of cell types like macrophages, neutrophils and natural killer cells and proteins such as the complement system. Innate immune cells can respond fast (sometimes within seconds) and their response remains unchanged upon a subsequent encounter with the same pathogen. This is in sharp contrast to adaptive immunity. Key players in adaptive immunity are T- and B-lymphocytes and humoral factors like immunoglobulins (antibodies). Although innate immune cells can respond to various conserved structures derived from microorganisms via so-called pattern recognition receptors (PRRs), lymphocytes are specific for one or a very limited number of particular antigens (Ags) that they recognize via their T- or B-cell receptor at the cell-surface [6]. Through recombination of particular gene segments (V(D)J recombination) a virtually indefinite number of B-cell receptors (cell surface immunoglobulins) and T-cell receptors can be generated. While B cells are activated following recognition of their cognate Ag in its native form, T cells require presentation of Ag as a peptide fragment in the context of major histocompatibility complex (MHC) molecules by antigen presenting cells (APCs). The activation of T- and B-cells induces clonal expansion of Ag specific effector cells that can mount an efficient immune response. Although adaptive responses in naive T- and B-cells are generated in a much slower fashion compared to innate responses and can take up to more than a week to fully develop, repeated exposure to the same Ag

induces a rapid 'memory' response that is enhanced both qualitatively and quantitatively.

T- and B-cells

T- and B-cells each make a unique contribution to immunity, but also interact with each other for optimal execution of their respective functions. B-cells support immune responses via production of various types of immunoglobulins with distinct function, *e.g.* neutralization of bacterial products [7, 8], opsonization of microorganisms [9] and 'barrier' formation at mucosal surfaces [10]. As for T-cells; two distinct types of T-cells exist: CD4+ helper T-cells (Th-cells) and cytotoxic CD8+ T-cells (CTLs). They contribute to immunity either through direct killing of target cells (CTLs) or via recruitment and activation of specific elements of the innate immune system capable to deal with a particular class of microorganisms (Th-cells). Different types of Th-cells have been identified. For long, a model proposed by Coffman and Mosmann [11], in which Th-cell differentiation was characterized by a binary fate choice has been in place. In this model, Th1 cells (characterized by the production of IFNγ) contribute to the response against intracellular microorganisms (*e.g.* certain bacteria species, viruses), while Th2 cells (producing IL-4, IL-5, IL-13) regulate immunity against parasites (*e.g*. helminths). Furthermore, Th1 and Th2 cells have been implicated as important contributors to several autoimmune diseases and asthma/allergies, respectively [12, 13]. However, the identification of two additional T cell subsets, regulatory T cells (Treg) and Th17 cells has resulted in a revision of the original Th1/Th2 paradigm [14]. Th17 cells are key producers of IL-17, a cytokine involved in the coordination of tissue inflammation via induction of other inflammatory cytokines, chemokines and matrix-metalloproteases as well as recruitment and activation of neutrophils [15-17]. In addition, IL-17 contributes to mucosal host defense [18] and immunity against extracellular bacterial and fungal pathogens [19]. Importantly, it has been suggested that Th17 cells play a crucial role in animal models of inflammatory bowel disease, rheumatoid arthritis and experimental autoimmune encephalitis, diseases that were formerly attributed to derailed Th1 responses [15, 20].

Regulatory T-cells

Although the effector arm of the immune system is indispensable for the protection against microorganisms, it needs to be tightly regulated in order to prevent excessive inflammatory responses. Even more importantly, unregulated responses can even lead to overt auto-immune disease in the genetically susceptible individual (**Table 1**). Regulatory T-cells (Treg) are crucial for prevention of these unwanted immune

responses. Several subsets of Tregs can be distinguished: intrinsic Treg and adaptive Treg. Intrinsic or naturally occurring Tregs originate in the thymus and depend on the expression of the transcription factor forkhead box p3 (Foxp3) for their development and function [21-23]. They become suppressive upon recognition of their cognate antigen and can inhibit proliferation and effector function of CD4+ and CD8+ effector T-cells in an Ag-independent, cell-contact dependent fashion [24, 25]. Genetic deletion or ablation of Tregs by targeting Foxp3 in mice leads to inflammatory disorders or catastrophic autoimmunity [26-28]. The importance of proper Treg function in humans becomes apparent in individuals carrying a mutation in Foxp3, that suffer from a rare syndrome (IPEX) characterized by uncontrolled lymphocyte responses leading to autoimmune enteropathy, dermatitis, thyroiditis, and neonatal onset of insulin-dependent type 1 diabetes mellitus [29, 30]. Likely, defective Treg function or numbers play a role in other immune disorders in the human setting as well [31]. Next to intrinsic Treg, the adaptive or induced Treg subsets, which can be generated in the periphery via several mechanisms, can exert their immunosuppressive function in a cell-contact dependent manner or via secretion of anti-inflammatory cytokines like IL-10 and TGFβ [32-34].

Table 1

Disease	Target organ/tissue	Main immune target(s)	Effect / complications
Type 1 diabetes mellitus	Pancreas (insulin secreting β-cells)	GAD65, (pro)insulin, IA-2	Insulin dependency, cardiovascular disease, nephropathy, retinal damage, neuropathy
Crohn's disease	Most often ileum and colon, but the entire gastro-intestinal tract may be affected	Commensal bacteria	patchy inflammation in gastrointestinal tract, abdominal pain, diarrhea, stenosis, fistulae, extra-intestinal complications (eyes, skin)
Systemic lupus erythematosus	Not specific	Host nuclear antigens (*e.g.* DNA and histone antigens)	rash, nephropathy, joint inflammation, pericarditis, neurological disorders, hematological disorders
Rheumatoid arthritis	Synovium of the joints (most often small joints of the hand and feet, but also larger joints can be involved)	Largely unknown, citrullinated peptides might be a target	joints: swelling, pain, deformity. Extra-articular: vasculitis, pericarditis, rheumatoid nodules

Target organs, main immune stimuli and complications of four prototypical auto-immune diseases. In the case of Crohn's disease, true autoantigens are probably not present, yet commensal bacteria might be considered as 'self'. Abbreviations: *GAD65*, glutamic acid decarboxylase 65; IA-2, islet antigen

Treg also modulate immune responses by downregulating Ag presentation and cytokine production by APCs [35-37]. In conclusion, Treg are key immune regulators that deploy several mechanisms to prevent excessive or unwanted immune responses.

Dendritic Cells

In contrast to the different cell types of the innate branch of the immune system that do not require prior “education” in order to respond to invading microorganisms, T- and B-cells need to be instructed in order to recognize their Ag and acquire their effector function. This key instructive role is carried out by a specialized antigen presenting cell (APC) called the dendritic cell (DC). First identified by Ralph Steinman in the early 70’s [38], DCs have now been widely accepted as the most potent APC, capable of inducing protective adaptive immune responses but also tolerance to self Ag [39-41]. DCs come in different flavors; the most obvious classification being a division in various subsets of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), which can be further subdivided on the basis of expression of distinct (cell surface) markers and functions. Importantly, mDCs and pDCs orchestrate different types of immune responses. The hallmark of pDCs is production of vast amounts of type I IFNs following activation with microbial nucleic acids [42] and these cells are suggested to play a crucial role in antiviral immunity. On the other hand, mDC subtypes have the capacity to respond to diverse microbial structures and play a role in directing T-cell differentiation, as discussed later. Due to the extremely low frequency in which mDCs and pDCs are present in blood and tissues, research using primary blood DCs is complicated. In stead, many studies focusing on (m)DC biology make use of monocyte-derived DCs, cultured from blood monocytes in the presence of IL-4 and GM-CSF, which share many of the phenotypic and functional characteristics of naturally occuring mDCs [43].

DCs are uniquely located at contact sites with the external milieu, such as the skin, vagina, gastrointestinal system and lungs. They are well-equipped to continuously monitor these tissues for the presence of microorganisms. For instance, in both the gut and lung, DCs send dendrites through the epithelial cell layer and directly sample the luminal surface while preserving epithelial barrier integrity [44-46]. Despite the fact that DCs are professional phagocytes like macrophages and neutrophils, they differ from these cell types in that they have developed means to 'preserve' useful information from the ingested particles that serve to initiate adaptive immune responses. Both phagosomal degradation and acidification are much lower in DCs than in macrophages or neutrophils resulting in conservation of antigenic peptides and their

increased presentation on major histocompatibility complex class (MHC) molecules [47].

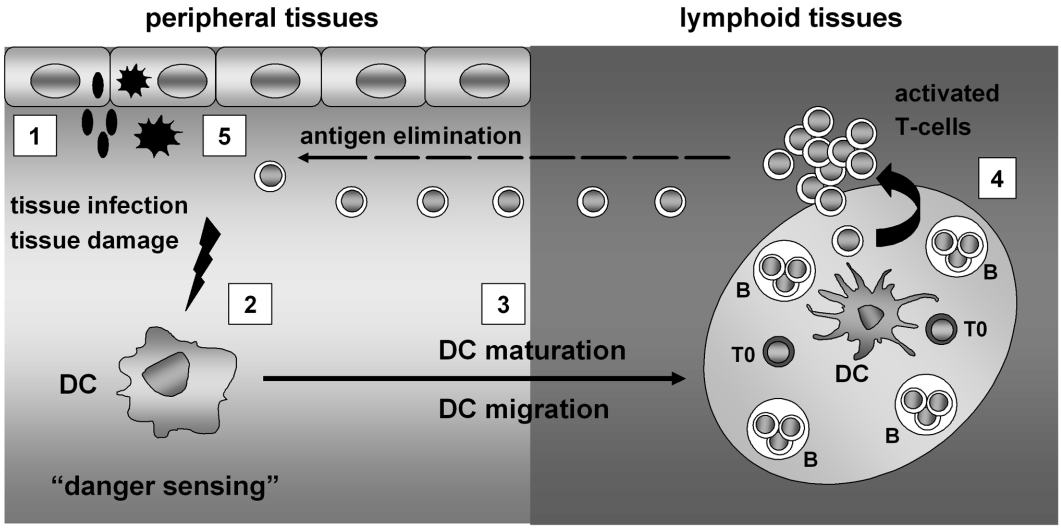
Both under steady state conditions [48, 49] and during infection, DCs migrate to secondary lymphoid organs draining the particular organ or tissue where they can interact with T- and B-cells (**Fig. 1**). Only following encounter with microorganisms or other 'danger signals', DCs undergo a process called maturation, which is characterized by differential expression of thousands of genes [50-52]. Mature DCs express high levels of MHC and costimulatory molecules such as CD80 and CD86, which enables potent induction of effector lymphocyte responses. DCs can activate both CD4+ T-cells and CD8+ T-cells and although many cell types have APC properties, DCs are superior in activating naive T cells. Importantly, while MHC-I-restricted Ag presentation is normally reserved for Ag derived from endogenous structures, DCs have the capacity to present Ags derived from both the intra- and extracellular milieu in the context of MHC-I. This process, called cross-presentation, enables efficient induction of CD8+ T-cells and is paramount for immunity against certain viruses and tumors [53]. The central role of DCs in orchestration of the immune response becomes apparent in murine models in which DCs can be transiently depleted *in vivo*. Herein, lack of DCs facilitates viral spread, impairs CD4+/CD8+ T cell and NK cell responses and results in increased morbidity and mortality as a result of viral infection [54, 55]. In the human setting, the contribution of DCs to adaptive immunity is underscored by their capacity to induce HIV-specific [56] or tumor-Ag specific T cell responses [57-59] following vaccination of DCs 'loaded' with viral or tumor Ag, respectively.

DCs and tolerance

Besides their role as key inducers of protective immune responses, it is now recognized that DCs play an equally important role in induction and maintenance of tolerance to self-Ag [60]. Tolerance induction does not appear to be specific for a certain DC subset or restricted to "classical" immature DCs but reflects a phenotype skewed towards expression of inhibitory signals (*e.g.* IDO, PDL-1, TRAIL, IL-10) rather than activation signals (*e.g.* CD80, CD86, OX40-L, IL-12). How DCs manage to induce immune responses to harmful stimuli, while preventing adverse reactions to innocuous Ag (especially in tissues with high Ag burden like the gut and lung) is still being explored. However, it has been shown that several subsets of mucosal DCs present at these 'immune challening' sites have the propensity to produce anti-inflammatory cytokines, like IL-10 [61-63]. In addition, DCs that migrate from the periphery in the absence of infection or other overt inflammatory signals generate T-cells with a suppressive

phenotype that induce tolerance *in vivo* [62, 64] or mediate self-reactive T-cell deletion [49, 65]. Importantly, despite the fact that mucosal DCs display a more regulatory phenotype, they can still mediate effector responses in case of invading pathogens, which may involve the same or different DC subsets as those responsible for tolerance induction [61, 66].

Figure 1



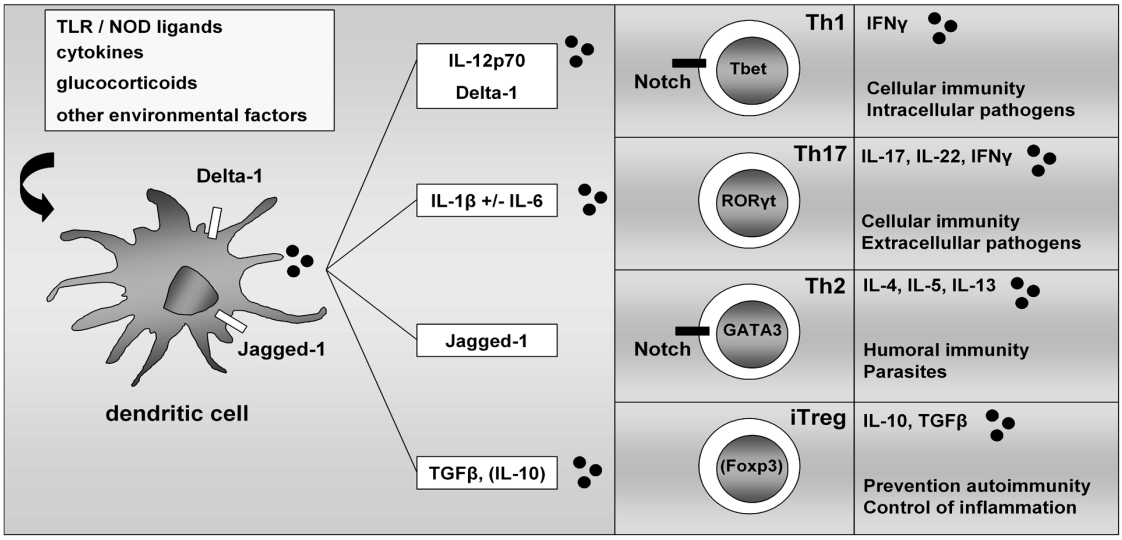
Schematic representation of DC function *in vivo*. DCs are present in virtually all tissues and organs and continuously monitor their environment for the presence of 'danger', *e.g.* invading microorganisms and tissue damage (1). Upon recognition of danger signals (2), DCs undergo a process called maturation and migrate to the lymphoid organs draining the particular organ or tissue (3). Here, DCs interact with and activate naive lymphocytes specific for their cognate Ag (4). The activated effector T-cells exit the lymphoid tissue and cause elimination of the peripheral threat (5). Under steady state conditions DCs contribute to immune homeostasis by deleting autoreactive T-cells and by inducing Treg.

DCs and T-cell differentiation

Next to T-cell activation, also differentiation of CD4+ Th-cells into different subsets that have the appropriate attributes to deal with a particular microorganism is regulated by DCs. Recent studies have provided more insight into how DCs decide which Th-cell subset needs to be generated. Signals by which a DC interprets its environment, mainly via binding to pattern recognition receptors (PRRs) and cytokine receptors, induce different 'DC programs'. These differently programmed DCs express particular cytokine profiles and cell surface molecules that relay, to naive Th-cells, the

properties of distinct lineages via repression or induction of transcriptional master regulators, namely T-bet (Th1) [67], GATA3 (Th2) [68] and RORγt (Th17) [69] (**Fig. 2**). For example, it is known that activation of DCs with LPS or flagellin can trigger release of IL-12p70 and thereby Th1 differentiation [70], while the pathways leading to Th2 induction are still less clear. For long it was thought that Th2 differentiation was a 'default' pathway when overt descriptive signals from the APC were lacking. However, it has been shown that interaction between Notch on T-cells and Jagged-1 on APCs (expressed following stimulation with cholera toxin or prostaglandin E2, two well know Th2 inducers) mediates Th2 differentiation [71, 72]. Polarization of naive T-cells towards the recently discovered Th17 subset can be induced upon IL-1β exposure of naive T-cells in the human setting, while IL-6 and IL-23 can potentially enhance their effector function [73-75], which is in contrast to the induction of murine Th17 cells by the combination of IL-6 + TGFβ [15, 76].

Figure 2



Schematic representation showing the central role of DCs in the decision process of Th-cell differentiation. DCs receive signals from their environment via for instance PRRs and cytokine-receptors, resulting in the induction of different 'DC programs'. These differently activated DCs can regulate naive T-cell differentiation via expression of particular cytokine profiles (●●) or cell-surface molecules (Jagged-1, Delta-1) that induce or repress key transcription factors involved in T-cell lineage commitment. This way, DCs organize the induction of an immune response that is apt to eradicate the specific microorganism involved or, in case of induction of inducible (i)Treg, to maintain homeostasis. Besides the beneficial actions of diverse T-cell subsets in fighting infection, deregulated actions of these effector cells contribute to the pathogenesis of diseases like type 1 diabetes, Crohn's disease, asthma and allergies.

The potential contribution of PRR activation on DCs to Th17 induction is still under investigation, but recent data suggest that activation of human DCs with the bacterial cell wall component peptidoglycan can support this process [74, 77].

Thus, DCs continuously interpret signals from the periphery, whether during homeostasis or infection and relay this information to cells of the adaptive immune system, thereby enabling induction of the appropriate immune response or maintenance of immune quiescence.

Pattern recognition receptors (PRRs)

As described above, DCs are uniquely positioned to readily sense invading microorganisms at key entry sites. Detection of microorganisms is based on the recognition of conserved molecular structures, termed pathogen-associated-molecular-patterns (PAMPs) via PRRs. PAMPs are ideal targets for the immune system, because they are (1) invariant among microorganisms of a given class, which enables recognition of multiple microorganisms using one receptor (2) preferentially expressed by microorganisms and not host cells and (3) are typically essential for microorganism survival, thus limiting the chance of immune evasion by modification of these structures. Importantly, both commensal and pathogenic microorganisms can express the same molecules and it has even been suggested that certain host structures [78, 79] can be recognized by PRRs (hence the name PAMPs is not ideal). How the immune system deals with discrimination between self and non-self regarding the type of response that needs to be induced remains enigmatic. Mechanisms like restricted PRR expression/localization [80, 81], (PRR mediated) anti-inflammatory effects of commensal bacteria [82, 83] and expression of regulatory molecules [84, 85] seem to be important contributors.

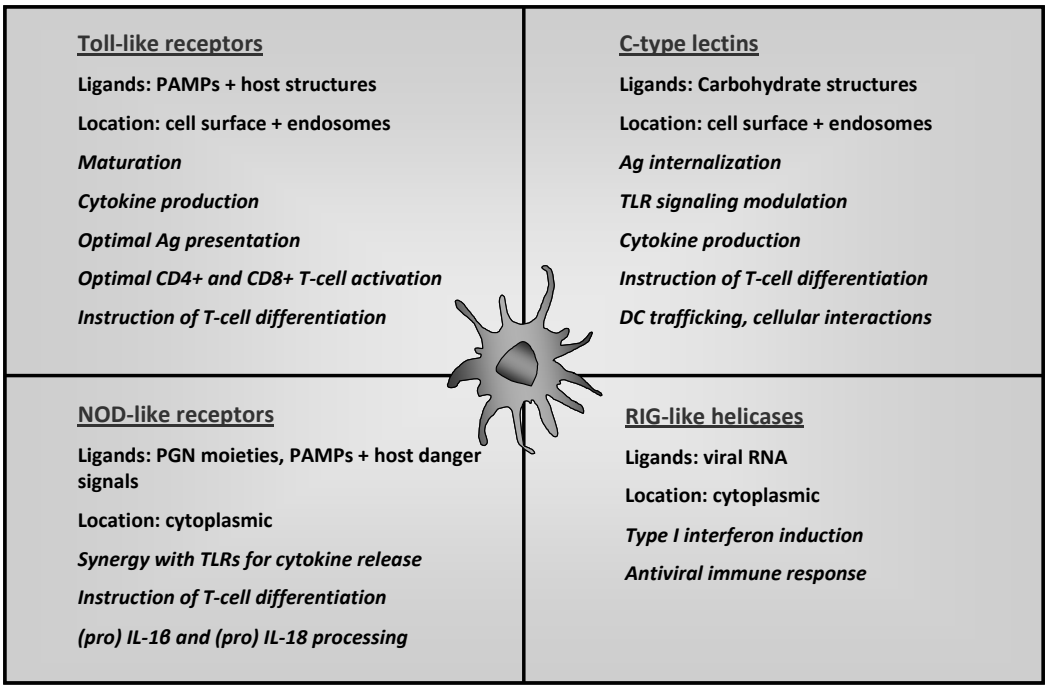
Toll-like receptors

Human DCs express various functionally distinct classes of PRRs (**Fig. 3**). The best characterized PRRs are the Toll-like receptors (TLRs). The TLR family in humans consists of 10 transmembrane members that are either located on the cell surface (TLR1, 2, 4, 5, 6 and 10) or within endosomal compartments (TLR3, 7, 8 and 9). The quest for identification of TLR ligands has yielded hundreds of, mostly microorganism-derived, structures, for example viral nucleic acids, components of bacterial and fungal cell walls, and bacterial DNA [86, 87]. In addition, it has been reported that endogenous ligands can activate TLRs. Recognition of structures associated with for instance tissue damage or cellular stress fits the “danger model” first proposed by Polly Matzinger

[88] that is based on the idea that the immune system is more concerned with entities that do damage than with those that are foreign. Among these endogenous ligands are molecules like fibrinogen [89], hyaluran [90], heat shock proteins [91, 92] and *high mobility group box-1* [93]. However, it should be realized that effects contributed to putative endogenous ligands could be due to possible contamination with microbial products such as endotoxins [94]. One illustrative example is the erroneous identification of TLR2 as the long-sought LPS signal transducer [95], which later turned out to be a result of the presence of TLR2-activating lipoproteins in the LPS used in that study [96]. Therefore, it is essential to carefully pinpoint the potential contribution of contaminating structures to the observed immune activating effects of endogenous ligands prior to translating recent findings into disease models or even therapeutic strategies.

TLR biology, an overview of TLR ligands and their contribution to inflammatory disorders are discussed in more detail in chapter 2.

Figure 3



General characteristics of the four main pattern recognition receptor families expressed by DCs. The most important effects of PRR triggering are given in italics. Abbreviations: *PAMP*, pathogen-associated molecular pattern; *Ag*, antigen; *PGN*, peptidoglycan

C-type lectins

C-type lectin receptors (CLRs) are a family of PRRs that specifically recognize carbohydrate structures, such as mannose, glycoproteins or β-glucans, enabling DCs to interact with a variety of pathogens and self-Ags. As for TLRs, expression of CLRs on DCs depends on activation state and the subtype studied; for instance, immature monocyte-derived DC express DC-SIGN, DEC-205, DCIR, Dectin-1 and mannose receptor (MR), while freshly isolated pDCs express mainly BDCA-2, Dectin-1 and DEC-205. An important function of CLRs is thought to be internalization of Ag for degradation in lysosomal compartments to enhance Ag processing and presentation [97]. In concordance with their Ag capture capacity, the expression of CLRs is generally higher on immature DCs compared to mature DCs. Ag that has been internalized via CLRs can be presented in the context of both MHC I and MHC II [98-100], enabling induction of both CD4+ and CD8+ T-cells. Besides Ag uptake, interaction between CLRs and self Ags expressed on both immune and non-immune cells can mediate other cellular functions, as has been reported for the interaction between DC-SIGN and ICAM-2 and ICAM-3 that regulates DC migration [101] and DC-T-cell interaction, respectively [102]. In addition, it is now clear that CLR binding directly activates several signaling pathways and can modulate TLR signaling. For instance, binding of the β-glucan receptor Dectin-1 can result in release of IL-6, TNFα and IL-23 or enhance TLR-induced IL-12p70 and TNFα production [103, 104]. Furthermore, CLR triggering on DCs can induce T-cell differentiation, as Dectin-1 stimulated DCs are capable of generating Th17 cells both *in vitro* and *in vivo* [103].

NOD-like receptors

The recently discovered NOD-like receptors (NLRs) constitute an ever growing family of structurally related PRRs. Unlike TLRs, they are soluble proteins that reside in the cytoplasm. The first NLRs discovered were NOD1 and NOD2 that recognize specific moieties of bacterial peptidoglycan (PGN) [105, 106]. Much of the recent research has focused on NOD2, owing to the association of mutations in this PRR with susceptibility to Crohn's disease (CD) [107, 108]. Members of the NALP subfamily constitute part of multiprotein complexes named inflammasomes that control processing and activation of key pro-inflammatory cytokines like IL-1β and IL-18, via activation of inflammatory caspases. Of clinical relevance is that mutations in NALP3 are associated with several inflammatory disorders, such as Muckle-Wells syndrome and gout [109].

The function of NLRs and their role in several inflammatory disorders are further discussed in Chapter 2.

RIG-like helicases

TLRs residing in the endosomal compartment are endowed with the capacity to induce immune responses following recognition of nucleic acids. However, since the ligand sensing leucine-rich-repeat domain of these TLRs is directed towards the interior of the endosomal compartment, they are incapable of sensing cytoplasmic nucleic acids. Hence, sensing of these structures, for instance upon viral infection of DCs, likely involves alternative PRRs. One prototypical virus-associated PAMP is double-stranded RNA (dsRNA), an intermediate formed during intracellular replication of many viruses. Two molecules that execute anti-viral host responses after recognition of cytoplasmic dsRNA are protein kinase R (PKR) and 2'-5'-oligoadenylate synthetases (2'-5'-OAS). PKR functions in anti-viral immunity via inhibition of (viral and host) translation [110], while 2'-5'-OAS are enzymes involved in activation of RNase-L [111], which limits spread of viral infection via cleavage of both cellular and viral RNA. Despite the fact that these effector molecules are important contributors to the antiviral immune response, they act in a secondary, type I IFN dependent fashion. Thus, accruement of a true 'antiviral' state requires stimulation via type I IFN. Two cytoplasmic RNA helicases; *retinoic-acid-inducible gene* (RIG-I) and *melanoma differentiation associated gene* 5 (MDA5) have recently been identified as crucial mediators of type I IFN production following recognition of viral RNA [112]. These molecules share two N-terminal *caspase recruitment domains* (CARDs) that allow interaction with downstream signaling molecules, and a C-terminal RNA helicase domain, used to bind distinct RNA species. A third helicase, called LGP-2, lacks CARD domains and is thought to function as a negative regulator, for instance by sequestration of dsRNA [113, 114]. When activated, RIG-I and MDA5 recruit the shared adapter protein *CARD adapter inducing IFNβ* (CARDIF, also called VISA, MAVS and IPS-1 [115-118]), which results in initiation of downstream signaling events and the activation of NFkB and *interferon regulatory factors* (IRFs) that cooperate in the production of type I IFNs. RIG-I and MDA5 respond to different RNA species and RNA viruses. RIG-I recognizes ssRNA containing 5'-triphosphate groups [119] and dsRNA or DNA/RNA duplexes with 3'- overhang [120] and mediates responses to several RNA viruses like paramyxoviruses, influenza virus and Japanese encephalitis virus [121]. On the other hand, MDA5 recognizes the synthetic viral dsRNA analogue poly(I:C) and is crucial for the response to several picornaviruses [121, 122]. Importantly, in some cases induction of antiviral responses, *e.g.* during infection with West Nile virus, involves both MDA5 and RIG-I [123]. With the recent identification of DAI (*DNA-dependent activator of IFN-regulatory factors*) [124], also the responses to cytoplasmic DNA are starting to be elucidated. Unquestionably, future research will reveal the existence of additional (cytoplasmic)

DNA and RNA sensors and increase our understanding of the organization of immune responses to nucleic acids.

PRR Cross-talk

Most *in vitro* studies focusing on the effect of PRR activation on DC biology have been performed using single agonists. However, microorganisms express multiple PAMPs and are thus likely to trigger different PRRs. Combined activation of particular TLRs results in distinct differences with respect to the intensity and nature of the response [125, 126]. For example, DCs can produce massive amounts of the Th1 polarizing cytokine IL-12p70, but do so only if triggered by selected combinations of TLR ligands. Optimal IL-12p70 release is achieved when additional signals (*e.g*. IFNγ or CD40L) are involved; implicating that interaction of DCs with other cells of the immune system can maximize the response. In addition to boosting IL-12p70 secretion, synergistic stimulation can cause profound upregulation of the Th1-promoting ligand Delta-4, while expression of the Th2 associated Jagged-1 is decreased, resulting in an increased and more sustained Th1 skewing capacity [51]. Interestingly, synergy seems to be specifically induced when at least one endosomal TLR is involved [51, 127, 128]. This suggests that a combinatorial 'security code' exists that licenses maximal cytokine release and effector T-cell generation only in DCs that have encountered and taken up microorganisms. In this respect, it is interesting to note that the efficiency of Ag presentation of phagocytosed cargo is dependent on the combined presence of Ag and TLR ligands within the same phagosomal compartment [129].

In addition to the synergy observed between PRR belonging to the same family, there is also cross-talk between members of different PRR families in DCs. Although it is well known that TLR activation in DCs generally results in profound cytokine release, stimulation of DCs with NOD1 or NOD2 ligands alone, does not induce significant cytokine production [130, 131]. Interestingly, simultaneous stimulation of NODs and TLRs enhances the release of pro-and anti-inflammatory cytokines by DCs in a synergistic fashion [130, 131]. Also pro-inflammatory cytokines such as TNFα or IL-32 increase NOD-mediated responses in human mononuclear cells [132, 133]. The mechanism underlying synergy remain largely unresolved, but might include downregulation of negative feedback molecules, stabilization of transcription factors [51] or increased expression of PRRs and their signaling components [134-136]. Importantly, triggering of multiple PRRs can also result in negative regulation. Examples are the reported suppression of TLR4-induced chemokine and cytokine production as a result of TLR2-induced IL-10 [137] and modulation of TLR-mediated DC

activation by DC-SIGN [138, 139]. This phenomenon might play a role in avoiding excessive inflammatory responses but can also be exploited by pathogens that can use PRR cross-talk as a way to tip the balance between pro-and anti-inflammatory cytokines [140, 141].

In conclusion, PRR cross-talk represents an important regulatory mechanism for the immunological response to pathogens *in vivo*. This phenomenon might have useful implications in the clinical setting where it can be exploited to maximize DC activation to improve DC vaccination strategies. On the other hand it can prove to be the immune systems Achilles' heel, if microorganisms use PRR cross-talk to skew immune responses to favor their survival.

Dendritic cells & Enteroviruses

Viral evasion strategies

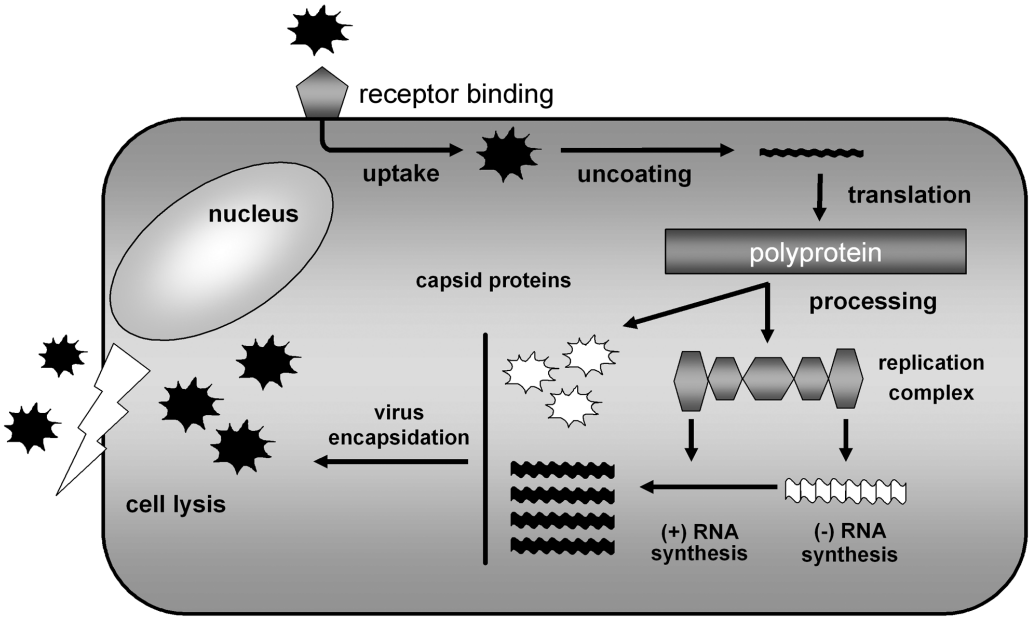
As discussed earlier, DCs play a crucial role in anti-viral immunity. However, viruses have co-evolved with their host in order to escape the immune system and establish a productive infection. The escape mechanisms used by viruses are numerous and include inhibition of anti-viral effector molecules [142, 143], cleavage of MDA5 or CARDIF [115, 144] and inhibition of type I IFN transcription [145]. Several viruses can also directly target DCs. Infection of DCs with *herpes simplex virus-1 (HSV-1)*, *vacciniavirus* or *cytomegalovirus* (CMV) can inhibit upregulation of costimulatory molecules and MHC molecules [146-148], affecting lymphocyte activation. In some cases, infection even leads to specific degradation of T-cell stimulating molecules like CD83 [149]. Other viruses exploit the capacity of DCs to instruct T-cell differentiation via the production of particular cytokine profiles. In case of *measles virus* and *human herpes virus 6*, inhibition of IL-12p70 production can hamper the induction of Th1 cells [150]. Other viruses, such as CMV, encode IL-10 homologues [151] that impair TLR-induced DC maturation and production of pro-inflammatory cytokines [152]. Besides the modulating effects that many viruses have on DC function, also decreased DC viability, for instance after infection with *herpes simplex virus-1* [153], might hamper proper induction of anti-viral immune responses.

Human enterovirus-B

Human enterovirus-B (HEV-B) serotypes like coxsackievirus-B (CVB) and echovirus (EV) are single-stranded RNA viruses of positive polarity that form a species within the enterovirus genus of the *Picornaviridae*. They are lytic viruses that enter the cell

through binding of specific receptors, e.g. *coxsackievirus-adenovirus receptor* (CAR) for CVBs [154, 155] or *decay-accelation factor* (DAF) for EVs [156]. Upon receptor binding, HEV-B viruses are invaginated into the cytoplasm and enter the cell via endocytosis, although the exact pathways leading to internalization or release of the virus into the cytoplasm remain largely unidentified. Once present inside the host cell, the virus is uncoated, viral RNA released into the cytoplasm and subsequently translated into a single polyprotein that can be proteolytically cleaved by virally encoded proteases into a group of capsid proteins and the nonstructural replication proteins. The nonstructural proteins form replication complexes that transcribe complementary negative-stranded RNA molecules from the positive-strand genome. The negative-stranded RNA can then serve as a template for the production of a large amount of positive-stranded viral genomes, which are translated until sufficient capsid proteins are present to ensure proper encapsidation of viral genomes, resulting in the formation of newly synthesized virus particles. When the host cell loses its integrity due to the consequences of viral infection (discussed below), the accumulated virus particles are released and a new infection cycle can occur (**Fig. 4**).

Figure 4



Schematic representation of the enterovirus life cycle.

Following infection, HEV-B proteins can modify a number of host cell functions, *e.g.* shutoff of host translation [157, 158], disruption of nucleo-cytoplasmic trafficking [159, 160] and inhibition of transport of newly synthesized proteins to the cell surface [161, 162]. These mechanisms can contribute to viral evasion of the immune system, as they can hamper release of cytokines [163] and transport of newly synthesized MHC molecules to the cell surface [161], which potentially disables induction of adequate T-cell responses.

HEV-B and human diseases; a link between HEV-B and type I diabetes mellitus?

HEV-B infections are common, with epidemics peaking between June and October in temperate climates. The outcome of infection is usually mild or even remains asymptomatic. However, in some cases infection can cause serious diseases such as aseptic meningitis and myocarditis [164, 165]. Also, there has been a long standing interest in HEV-B infections as environmental causes of type I diabetes mellitus (T1DM). T1DM is a prototypical autoimmune disease that is characterized by progressive loss of insulin producing β-cells in the pancreas, due to the actions of auto-aggressive T-cells. The association between T1DM has been studied ever since it was recognized that CVB antibodies were found more frequently in patients with TIDM than in healthy controls [166]. In addition, HEV-B RNA is found at higher frequencies in the blood of diabetic subjects [167]. Although several mechanisms, such as direct infection of pancreatic β-cells [168, 169] or molecular mimicry between viral and islet Ags [170] have been proposed, there is still no consensus on whether or not a causal relationship between HEV-B infection and T1DM exists. Virtually no data is currently present on the potential effects of HEV-B infection on human DC function, which is surprising taking into account the crucial role of DCs in regulation of T-cell responses and the potential effects of exposure to viral structures on DC activation. It is tempting to speculate that DCs might somehow provide a 'missing link' in the relation between HEV-B and T1DM.

Aim & outline of this thesis

The research described in this thesis has been performed to gain more insight into the function of human DCs in health and disease with a particular focus on the role of PRRs in DC biology.

Chapter 2 of this thesis provides a concise overview of the biology of two important PRR families; the Toll-like receptors and the NOD-like receptors and discusses their contribution to a variety of inflammatory disorders, like rheumatoid arthritis, Crohn's

disease and a number of rare auto-inflammatory diseases. In **Chapter 3** the expression profiles of multiple PRR families in DCs under pro- or anti-inflammatory conditions was explored. Furthermore, we show that expression of different PRRs responding to viral RNA (both TLRs and cytoplasmic RNA sensors) is highly dynamic and differs significantly between distinct DC subsets. **Chapter 4** focuses on the consequences of the Crohn's disease related 3020insC frameshift mutation in NOD2 on DC function. We show that DCs derived from donors homozygous for this mutation fail to respond to the NOD2 ligand MDP, as determined by lack of cytokine production and impaired maturation. Furthermore, these DCs displayed an abrogated synergy for the release of both pro- and anti-inflammatory cytokines following combined stimulation with TLR and NOD2 ligands. The involvement of NOD2 in the production steps of one of the most potent pro-inflammatory cytokines, IL-1 β, is explored in **Chapter 5**. In **Chapter 6**, we investigated the susceptibility of DCs for infection with a variety of HEV-Bs and show that human DCs can be productively infected with several echoviruses, but not with closely related coxsackieviruses. Infection with echovirus resulted in rapid inhibition of TLR-mediated DC activation and cell death. **Chapter 7** provides evidence that DCs can engage a state of antiviral resistance following phagocytosis of virus-infected cells. This antiviral state, which is dependent on intact endosomal acidification and the presence of RNA within phagocytosed infected cells, can protect DCs against a lethal echovirus infection. These data might provide a mechanism by which DCs protect themselves against viruses when attracted to an environment with ongoing infection. Finally, **Chapter 8** discusses the results and significance of the findings described in this thesis.

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Chapter 2

Closing in on Toll-like receptors and NOD-LRR proteins in inflammatory disorders

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Future Rheumatology (2006) 1(4): 465-479

Summary

Our immune system faces the intricate task of eliminating pathogenic microorganisms, while auto-immunity against self-components or intestinal flora needs to be prevented. This complex task demands an exceptional level of regulation and specificity. Hereto, immune cells are equipped with a myriad of surface receptors, including cytokine-receptors and pathogen recognition receptors (PRRs). In this review we describe two important PRR families, Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-leucine rich repeat (NOD-LRR) proteins, and their crucial role in instruction of the immune response. Interestingly, these key immune-receptors have recently been identified as major players in several immune disorders, such as systemic lupus erythematosus (SLE), Crohn's disease (CD), auto-inflammatory diseases and rheumatoid arthritis (RA). These findings, and their implications for future research and therapy of immune disorders, will be discussed.

Toll-like receptor function and signaling

Our immune system is well-equipped to recognize specific, highly conserved structures unique to microorganisms of a given class. These so called pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), such as C-type lectins [1], members of the NOD-LRR family [2] and TLR [3]. TLRs owe their name to the *Drosphila* protein Toll. Toll was initially studied for its involvement in embryonic dorsoventral axis formation. However, $Toll^{-/-}$ animals quickly succumbed to massive fungal infection, implying a role for Toll in immunity [4]. Subsequent bioinformatics analyses led to the identification of mammalian TLRs. To date, 13 TLRs have been defined. However, certain TLRs can form heterodimers, which further increases TLR diversity. TLRs are type 1 transmembrane receptors, that recognize their ligands via a conserved C-terminal LRR domain. For some TLRs, ligand binding is dependent on the presence of accessory molecules [5, 6]. Many pathogen-derived structures, such as lipopolysaccharide (LPS), bacterial lipoproteins and double-stranded RNA are sensed by distinct TLRs (**Table 1**). In addition, it has been suggested that several endogenous structures –mostly associated with cell death, tissue damage or infection- can activate TLRs, like heat shock proteins [7], β-defensins [8] and fibrinogen [9]. However, these findings should be approached prudently, as contamination with for instance endotoxins could lead to serious misinterpretation of results [10, 11]. All members of the TLR family share a cytoplasmic domain called Toll/interleukin(IL)-1 receptor (TIR) domain, for its homology with the respective region of the IL-1 receptor. Activation of TLRs induces the recruitment of adaptor proteins that serve as a scaffold for downstream signaling molecules. The adapter protein myeloid differentiation factor (MyD)88 is involved in signaling of most TLRs, with the exception of TLR3, that signals via TRIF (Toll/IL-1 receptor domain-containing adapter-inducing IFN-β). The use of different adaptor proteins –and thus activation of distinct signaling pathways- brings specificity to TLRs and directs the type and magnitude of the immune response. While TRIF effectively activates interferon regulatory factor (IRF)3, resulting in IFN-β production [12, 13], MyD88 mediates transcription of cytokines and chemokines via NFkB and MAPK pathways. Interestingly, the recently discovered adapter proteins Mal (MyD88 adapter-like) [14] and TRAM (TRIF-related adapter molecule) [15] add to the further diversification of the TLR signaling network. To avoid excessive inflammation or auto-immunity following TLR activation, feedback mechanisms are of paramount importance. Indeed, a plethora of regulatory molecules and circuits are in place to control TLR responses. For instance, SIGIRR (single immunoglobulin IL-1 receptor-related protein) attenuates the

recruitment of receptor-proximal signaling components [16], while ST2 can prevent the translocation of NF-κB to the nucleus [17].

Table 1

TLR	LIGAND	SOURCE
TLR1+TLR2	Triacyl lipopeptides[a]	Bacteria
	Lipoarabinomannan[a]	Mycobacteria
	PorB porin[a]	Neisseria meningitidis
	Nystatin[121]	anti-fungal drug (*Streptomyces noursei)*
TLR2	Lipopeptide/lipoprotein[a]	Bacteria
	Lipoteichoic acid[a]	Gram-positive bacteria
	Lipopolysaccharide[a]	*P. gingivalis, L. interrogans*
	Lysophosphatidylserine[a]	*S. mansoni*
	HSP60[a], HSP70[a], HSPB8[122]	Host*, H. pylori*
	HMGB1[a]	Host
	Biglycan[123]	Host
TLR2+TLR6	Diacyl lipopeptides[a]	*Mycoplasma*
	Lipoteichoic acid[a]	*Staphylococci, Streptococci*
	GPI[a]	*P. falsiparum*
TLR3	dsRNA[a]	West-Nile Virus, Cytomegalovirus, *S. mansoni*
	siRNA[a]	Synthetic
	mRNA[a]	Host
TLR4	Lipopolysaccharide[a]	Gram-negative bacteria
	Taxol[a]	plants
	Anthrolysin O[a]	*Baccilus anthracis*
	Phosphorylcholine[a]	Filarial nematode
	HSP60[a], HSP70[a]	*Chlamydia pneumoniae,* Host
	Beta-defensin 2[a]	Host
	Fibrinogen[a]	Host
	Hyaluronic acid[a]	Host
	Fatty acids[a]	Host
	Modified LDL[a]	Host
	Biglycan[123]	Host
	Levan[124]	Host
	Heparan sulfate[125]	Host
TLR5	Flagellin[a]	Bacteria
TLR7	ssRNA[a]	Influenza, HIV-1, Parechovirus 1
	Imidazoquinoline[a]	Synthetic
	Loxoribine[a]	Synthetic
TLR8	ssRNA[a]	Coxsackie B virus, Parechovirus 1
	Imidazoquinoline[a]	Synthetic
TLR9	CpG DNA[a]	Bacteria, synthetic, DNA-viruses
	Hemozoin[a]	*P. falsiparum*
	Chromatin-IgG complexes[a]	Host
TLR10	Not determined[a]	
TLR11	Profilin-like molecule[a]	*Toxoplasma gondii*

Overview of TLR ligands and sources. Adapted from Isshi *et al [126]*, as indicated with (a), supplemented with most recent data from literature. Abbreviations: HSP, heat shock protein; HMBG, high mobility group box; GPI, glycosyl-phosphatidylinositol; dsRNA, double-stranded RNA; siRNA, short interfering RNA; mRNA, messenger RNA; ssRNA, single stranded RNA

Finally, the MyD88 splice-variant MyD88s can serve as a 'decoy' adaptor molecule that prevents the recruitment of IRAK-4 and subsequent downstream signaling [18].

In conclusion, TLRs play a central role in the response to 'danger' signals, such as the presence of microorganisms or tissue damage. Triggering of TLRs leads to activation of highly complex and intertwined signaling pathways that control immunity.

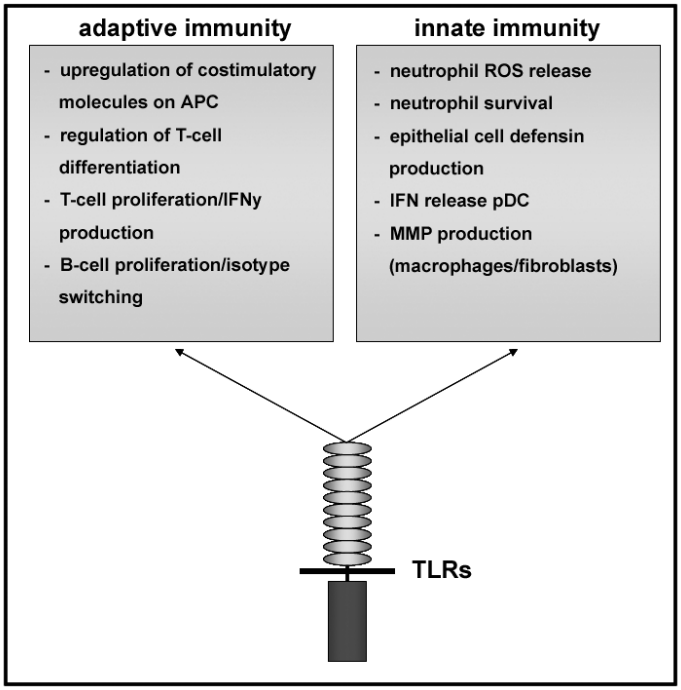
TLR expression and location

Different combinations of TLRs and TLR signaling molecules are expressed in various cell types involved in induction of innate immune responses. Monocytes, macrophages and neutrophils are all equipped with their own subset of TLRs enabling them to execute their effector function. However, TLR expression is not limited to innate immune cells, as they can also activate T- and B-cells and non-immune cells located at the interface of the interior and exterior milieu (**Fig 1**). For example, airway epithelial cells produce inflammatory cytokines and anti-microbial peptides in response to PAMPs or pathogens in a TLR-dependent manner [19-21].

The expression of TLRs is dynamic and can be increased by pro-inflammatory cytokines like TNFα, IFNγ and IL-6, which contributes to a higher sensitivity for TLR ligands and amplification of the inflammatory response [22-24]. On the other hand, TLR protein expression is tightly controlled via ubiquitin-mediated protein degradation [25]. Furthermore, prolonged exposure to microbial components results in hyporesponsiveness, as a result of decreased TLR expression and the upregulation of endogenous inhibitory molecules, a mechanism to prevent disproportionate inflammation [26]. TLRs also differ in their location within a cell. Whereas TLR1, -2, -4, -5 and -6 are present on the plasma membrane, TLR3, -7, -8 and -9 reside mainly within the endosomal/lysosomal compartment. The differential sub-cellular location of TLRs relates to the nature of the ligands that are recognized. Thus, TLRs at the cell surface mainly respond to extracellular pathogens, while intracellular TLRs are triggered by nucleic acids from bacteria and viruses, mostly following uptake of pathogens or pathogen-infected cells. In addition, the intracellular localization avoids unwanted and potential harmful inflammatory responses against extracellular self-DNA or mRNA released from dying or dead cells. Likewise, the polarized expression of TLR5 in intestinal epithelial cells prevents inflammatory responses to commensal bacteria in the intestine. TLR5 is absent on the luminal side of the cell and is only triggered by its ligand flaggelin (the PAMP in flagella from motile bacteria), once the epithelial barrier is breached by pathogenic bacteria [27, 28].

In summary, TLR expression in various tissues and cell types is dynamic and can be increased by several pro-inflammatory cytokines. Tissue- or cell-type specific localization of TLRs is organized such that instant recognition of invading pathogens is ensured, but responses to self-antigens are avoided.

Figure 1



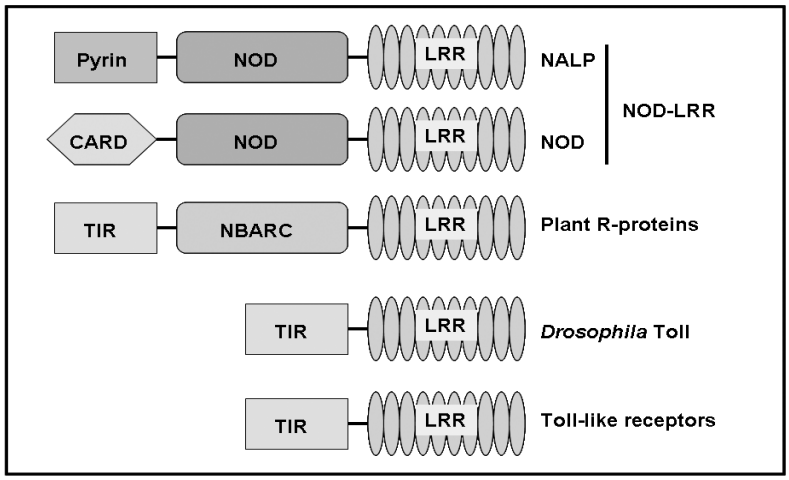
Effect of TLR triggering on innate and adaptive immune responses. *APC*: antigen-presenting cell; *DC*: dendritic cell; *IFN*: interferon; *MMP*: matrix metalloprotease; *ROS*: reactive oxygen species; *TLR*: Toll-like receptor

Role of TLRs in adaptive immune response

Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system and direct the type and course of an immune response. DCs can initiate immune responses against pathogens or tumors, but also prevent (auto-)immune responses harmful to the host. Immature DCs are present in virtually all organs and tissues and they continuously sample their environment for the presence of microorganisms.

Captured antigens are processed into peptides and subsequently presented to lymphocytes in lymph nodes. DCs can also activate natural killer cells and produce interferons, thus linking the innate and adaptive immune system [29]. DCs express a broad repertoire of TLRs and activation of these receptors results in upregulation of MHC and costimulatory molecules, and migration of DCs to the secondary lymphoid organs, where they activate naive T cells. The nature of the signals DCs receive, especially through TLRs and cytokine receptors, determines the type of immune response induced. Unstimulated DCs or DCs receiving immune-inhibitory signals, induce immune tolerance. On the other hand, triggering of TLR4 on DC results in Th1 polarization, while TLR2 activation rather supports a Th2 response [30, 31]. These findings have significantly contributed to our understanding of the classical Th1/Th2 paradigm and indicate that the combination of TLRs activated upon pathogenic encounter play a pivotal role in directing the type of immune response against that particular pathogen [32]. However, this phenomenon can sometimes be exploited by microorganisms. For example, *Candida albicans* is shown to induce Th2 or Treg differentiation via production of cytokines like IL-10, thereby preventing the Th1 response necessary for its eradication [33].

Figure 2



Structural organization of the NOD-LRR family members, as well as their homologues, the plant R proteins. Both mammalian and plant proteins possess a C-terminal ligand-sensing LRR domain, a central oligomerization domain (called NBARC in R proteins) and an N-terminal effector domain. Likewise, *Drosophila* Toll and mammalian Toll-like receptors contain a C-terminal LRR-domain and an N-terminal TIR effector domain, that binds intracellular adapter proteins (*e.g.* MyD88), which initiates signaling. *CARD*: caspase recruitment domain; *LRR*: leucine rich repeat; *NBARC*: nucleotide binding domain shared by Apaf-1, R proteins and CED-4; *NOD*: nucleotide oligomerization domain; *TIR*: Toll-interleukin-1 receptor

The NOD-LRR protein family

Recently, new LRR-containing PRRs have been identified and collectively christened the NOD-LRR family [2]. NOD-LRRs have a high structural homology to plant R proteins (**Fig 2**), that are involved in innate immune defense [34]. NOD-LRRs reside in the cytosol and are thought to be involved in sensing the presence of intracellular microorganisms. All NOD-LRR proteins share a C-terminal LRR domain, a central NOD domain -used to form dimers or oligomere complexes- and a distinct N-terminal effector domain. The NOD-LRR family can be divided into four distinct subfamilies: the CIITA, ICE-protease activating factor (IPAF), nucleotide-binding oligomerization domain (NOD) and NALP subfamilies, the latter two being discussed in more detail.

NOD1 and NOD2 respond to distinct moieties of the bacterial cell wall component peptidoglycan [35-38]. NODs are expressed in monocytes, macrophages and DCs, but also in non-hematopoietic cell types, like intestinal epithelial cells. Triggering of NODs results in homodimerization, the recruitment of adapter protein RICK via homotypic caspase recruitment domain (CARD)-CARD interactions and induction of subsequent signaling events [39]. Triggering of endogenous NODs leads to only a moderate secretion of cytokines through activation of NFκB and MAPKs. However, recent data indicate that NOD stimulation strongly synergizes with TLR-triggering for the production of both pro- and anti-inflammatory cytokines [40, 41]

Another NOD-LRR subfamily is the NALP family, consistent of 14 proteins that share structural similarity with the NODs [42]. In stead of an N-terminal CARD domain, NALPs display a so-called Pyrin domain. Certain NALPs, especially NALP3 (Cryopyrin/CIAS1/PYPAF), have recently been discovered as key regulators of the inflammatory cytokine IL-1β [43]. Several NFκB-activating signals induce production of the inactive pro-IL-1β protein. The processing of pro-1β into active IL-1β is mediated by activation of a multi-protein complex referred to as the *inflammasome*, of which NALPs and pro-inflammatory caspases are crucial components [44]. This activation of the inflammasome can occur independently of TLRs. Binding of NALPs to ASC (apoptosis-associated speck-like protein containing a CARD) leads to recruitment and activation of caspase-1 (also known as ICE: IL-1β converting enzyme) and production of bioactive IL-1β. Since NALPs posses LRR domains similar to that of TLRs, they are thought to play a role in pathogen recognition as well. Recent studies by the Tschopp and Núñez groups identified several compounds that can trigger NALP pathways, like the NOD2 activator MDP [45], bacterial RNA, imidazoquinolines (that also activates TLR7/8) [46] and gout-associated uric acid crystals [47]. However, it remains to be determined if these structures directly bind and trigger NALPs, or whether associated

factors are required for their activation. Future research will undoubtedly lead to the identification of other NALP ligands, similar to the ever growing list of TLR ligands that has been discovered in recent years. In parallel with regulation of TLR signaling, the presence of endogenous inhibitors like pseudo-ICE, ICEBERG [48] and PI-9 [49], that interfere with IL-1β processing, is crucial to prevent disproportionate inflammatory responses.

TLRs and NOD-LRR in immune disorders

Systemic Lupus Erythematosus

Since TLRs are such potent inducers of both innate and adaptive immune responses, they are implicated in many immune-disorders, such as CD [50, 51] and type I diabetes mellitus [52]. The clearest example is the relationship between TLR9 and the auto-immune disease systemic lupus erythematosus (SLE). SLE is thought to be related to defective clearing of apoptotic cells and characterized by chronic inflammation initiated by deposition of immune complexes (IC) in target organs [53]. The ICs consist of antibody/dsDNA and antibody/nucleoprotein complexes and cause release of a variety of chemokines and cytokines, attracting for instance neutrophils and DCs. *In vitro* stimulation with serum or purified DNA-IC from SLE patients results in significant IFNα production by a rare subpopulation of DCs, referred to as plasmacytoid (p)DCs [54]. It has now been determined that IFNα release by pDC is induced in a TLR9 and FcR dependent manner [55]. It was found that DNA-ICs are taken up via FcγRIIa, translocate to acidic lysosomes, where binding of DNA to TLR9 triggers the production of IFNα. Antibodies against FcγRIIa or addition of chloroquine, a known inhibitor of TLR9 signaling, both efficiently blocked IFNα production following stimulation of pDC with DNA-ICs [56]. An excellent review dealing with the complex interplay of these receptors in SLE has recently been published [54]. The finding that polymorphisms in FcγRIIa are correlated with susceptibility to SLE further supports a role for this receptor in delivery of complexes into the cell. So far, an association between TLR9 polymorphisms and susceptibility to SLE has not been established [57]. In stead, it has been found that polymorphisms in TLR5 are associated with protection from the development of this disease [58], via a mechanism that is currently unknown.

These data indicate that cooperation between seemingly distinct receptors, including TLRs, contributes to the perpetuation of the inflammatory response against DNA and/or nuclear proteins that is characteristic of SLE.

Crohn's Disease

Crohn's disease (CD) is a chronic Th1-mediated inflammatory disease that can affect any part of the gastrointestinal tract. CD is a multi-factorial disease, with a strong genetic component and a role for environmental factors. It is generally believed that the disease results from an exaggerated immune response directed against the normal intestinal flora. This idea is supported by the finding that animals that are kept in a germ-free environment generally do not develop colitis [59] The vast number of bacteria in the gut represents a plethora of potential PRR activating structures, and this situation demands a tight immune regulation. Both TLRs and NOD-LRR proteins play an important role in intestinal inflammatory responses. In the mouse colitis model of dextran sulfate sodium (DSS), TLR9 stimulation with DNA derived from luminal bacteria results in elevated proinflammatory cytokine and chemokine production and more pronounced histopathological damage in the colonic mucosa in wildtype mice, but not $TLR9^{-/-}$ mice [60], indicating that TLR9 signaling can contribute to intestinal inflammation. Similar responses were obtained after TLR5 activation using flagellin [27]. Interestingly, flagellin is not only recognized by TLR5. Cytosolic flagellin can activate IPAF, a NOD-LRR protein, which results in production of IL-1β [61, 62]. The availability of two sensory pathways for flagellin potentially enables regulation of the intensity of the immune response, depending on the virulence of the pathogen encountered. Interestingly, TLR2 agonists do not cause an inflammatory response in the DSS colitis model [27], an effect that might be related to the induction of a Th2 cytokine profile following TLR2 triggering. Despite the fact that MyD88-dependent TLR5 and TLR9 responses can contribute to colonic inflammation, it was recently found that MyD88-deficient mice exhibit an increased susceptibility to DSS induced colitis [63]. This would suggest a protective role for MyD88 dependent signaling in preventing colonic inflammation.

Also on genetic level TLRs and NOD2 have been linked to CD. For instance, the TLR4 Asp299Gly polymorphism, that is associated with increased susceptibility to Gram negative infections, is found more frequently in CD patients than in the healthy population [50, 51]. However, the most compelling evidence is found for polymorphisms in NOD2. Especially the 3020insC frameshift mutation in NOD2 that results in a truncated form of the protein is highly associated with CD [64, 65]. Although several mouse models have suggested that this mutation results in enhanced NFkB activation and IL-12 or IL-1β production [66, 67], virtually all studies done with human cells point towards a loss-of-function mutation, resulting in defective recognition of MDP and loss of synergy between TLRs and NOD2 [41, 68, 69]. In

addition, NOD2 KO mice were found to have decreased expression of a subgroup of anti-microbial peptides [70]. How exactly a loss-of-function mutation in NOD2 leads to an unwarranted immune response towards intestinal flora is not completely clear yet, but might be related to defective immune regulation by impaired release of immunosuppressive cytokines, such as IL-10 [41, 68, 69]. Besides its involvement in CD, NOD2 was recently identified as the susceptibility gene for another granulomatous disorder, called Blau syndrome (BS) [71]. BS is a rare disease that features early-onset granulomatous arthritis, uveitis, skin rash and camptodactyly (Table 2). These data indicate that different mutations in NOD2 can have distinct physiological consequences. In the case of BS, the NOD2 mutations all affect the NOD domain of the protein [71, 72]. Since the NOD domain is not reported to be involved in the recognition of PAMPs, these mutations may cause ligand-independent NOD2 activation and inflammation, which is also observed in other so-called auto-inflammatory disorders, discussed below.

NALPs and auto-inflammatory syndromes

Auto-inflammatory diseases are characterized by recurrent episodes of seemingly unprovoked systemic inflammation that, unlike autoimmune disease, lack high-titer antibodies or the involvement of antigen-specific T cells. Mutations in NALP3 have unquestionably been linked to three prototypical auto-inflammatory syndromes: familial-cold auto-inflammatory syndrome (FCAS) [73], Muckle-wells syndrome (MWS) [73] and neonatal-onset multisystem inflammatory disease (NOMID) [74, 75], and are now considered to be a continuum of one disease, referred to as cryopyrin-associated periodic syndromes (CAPS). The CAPS share features like episodes of fever, arthritis and increased levels of inflammatory markers (**Table 2**). Most NALP3 mutations affect the NOD domain of the protein and it has been hypothesized that they interfere with auto-inhibitory LRR-NOD interaction and cause NALP3 inflammasome activation -and IL-1β production- even in the absence of NALP3 activating structures [43]. In line with this, macrophages from MWS patients were found to spontaneously secrete IL-1β [43]. The role of IL-1β in these diseases is further supported by the finding that treatment with the IL1-R antagonist Anakinra results in dramatic improvement of clinical symptoms and laboratory markers of inflammation in patients with MWS, FCAS and NOMID [76-78].

Table 2

Disease	Symptoms	Affected gene	Reference
MWS	episodes of rash, arthralgia, fever, conjuctivitis, frequent sensorineural hearing loss	NALP3	[73]
FCAS	cold induced episodes of rash, arthralgia, fever, conjuctivitis	NALP3	[73]
NOMID	rash, papilledema, uveitis, hepatosplenomegaly, sensorineural hearing loss, arthropathy, epiphyseal boneformation	NALP3	[74, 75]
Blau Syndrome	granulomatous papular rash, uveitis, iridocyclitis, granulomatous arthritis, camptodactyly	NOD2	[71, 72]

Overview of members of the NOD-LRR family involved in systemic auto-inflammatory disorders with rheumatic manifestations. FCAS: familial cold autoinflammatory syndrome; LRR: leucine rich repeat; MWS: Muckle-Wells syndrome; NOD: nucleotide-binding oligomerization domain; NOMID: neonatal-onset multisystem inflammatory disease

TLRs, NOD-LRRs and their involvement in joint inflammation

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease that is characterized by inflammation of the synovial joints leading to cartilage degradation and bone destruction, affecting approximately 1% of the population worldwide. The first evidence that suggested RA to be an autoimmune condition originated from the association of RA with certain human leukocyte antigen (HLA) subtypes [79]. Secondly, various autoantigens, including rheumatoid factor and citrullinated peptides, were found to be associated with susceptibility to and severity of RA [80, 81]. Despite multiple efforts, the precise role of HLA subtypes and autoantigens in RA pathogenesis remains to be elucidated. Nowadays, strong evidence shows a pivotal role for activated inflammatory cells in the initiation and perpetuation of the disease and it is now generally accepted that APCs are key players in the pathogenesis of RA. The disease process of RA can be divided in several stages. During the early stages, synovial cell proliferation, infiltration of proinflammatory cells and defects in apoptosis lead to synovial thickening. As the disease process develops, the inflamed synovium invades the surrounding cartilage and bone, leading to complete joint destruction. Although the exact mechanism underlying RA pathogenesis is still unknown, the involvement of microorganisms has often been suggested. The fact that DNA viruses, including Epstein-Barr virus (EBV) [82], Cytomegalovirus (CMV) [82] and parvovirus [83] have been shown to be present in RA synovium and fluid, supports this hypothesis. Likewise, several reports suggest the possible involvement of bacterial products in the

inflammatory circle of RA [84, 85]. Although a direct link between the presence of pathogens and RA has not been demonstrated thus far, the recent identification of TLRs sheds new light on the involvement of PAMPs in the articular inflammatory response.

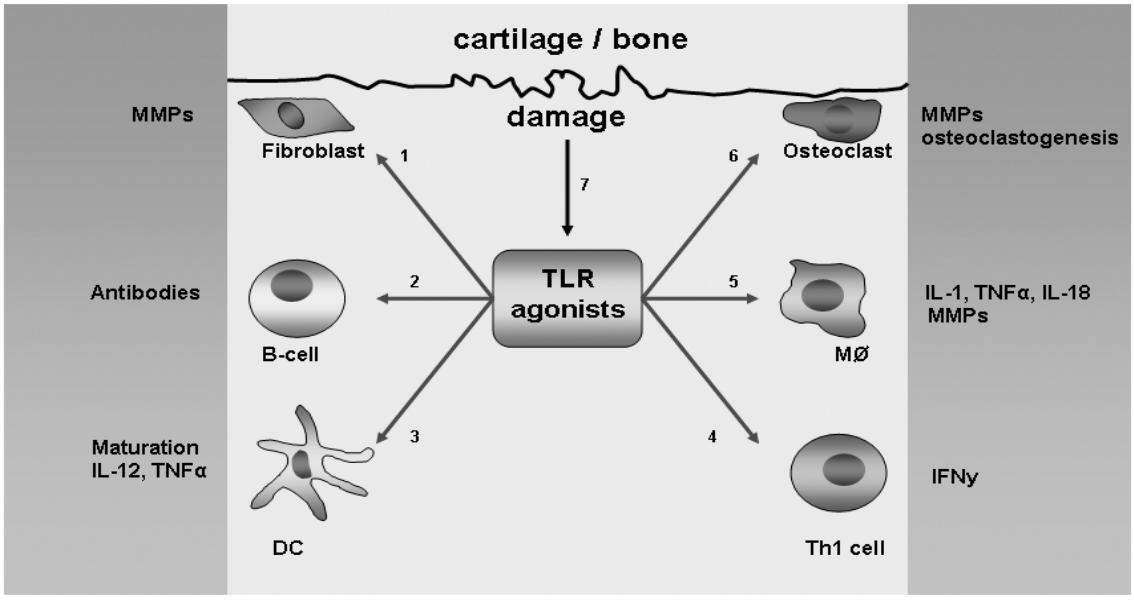
TLRs in RA initiation and perpetuation

Interestingly, many animal models of arthritis have applied TLR agonists for the induction of arthritis far before TLRs had been identified. More recent studies have substantiated a role for TLRs in initiation of experimental arthritis. For instance, TLR2 was found to play an essential role in the induction of streptococcal cell-wall induced arthritis since both $TLR2^{-/-}$ and $MyD88^{-/-}$ mice showed reduced signs of inflammation compared with control mice [86]. In addition, $TLR4^{-/-}$ mice have been shown to be less susceptible to collagen-induced arthritis [87] and ST2, an endogenous TLR4 inhibitor, attenuates disease severity in the same animal model [88]. Though informative, it should be realized that these animal models are rather artificial and do not provide an explanation for the role of TLRs in the pathogenesis of RA in the human setting.

To determine whether TLRs are involved in the initiation and/or perpetuation of RA in humans, is extremely difficult. It is particularly complicated by the fact that the disease process is likely to have started far preceding the onset of symptoms, as demonstrated by the presence of citrullinated auto-antibodies and inflammatory markers. Nevertheless, a vast body of evidence links TLRs to the pathogenesis of RA. First, the expression of TLRs in synovial tissue from RA patients is increased compared to the expression found in osteoarthritis patients or healthy controls [89]. Secondly, DCs from RA patients are highly responsive to TLR ligands and produce higher levels of pro-inflammatory cytokines compared to control DCs [90]. Likewise, various groups have demonstrated that triggering of selective TLRs results in an increased production of chemokines in RA patients [91]. Taking into account that TLRs respond to endogenous danger signals, combined with the general belief that a variety of those structures - such as heat shock proteins, RNA from necrotic cells and hyaluronic acid- are present in the synovial compartment, further supports the involvement of TLRs in RA. In addition, RA patients have high levels of circulating ICs and it was recently found that binding of IC to FcγRII can facilitate TLR7 [92] and TLR9 [93] mediated B-cell activation, a mechanism that is likely to function in DCs and macrophages as well. Interestingly, activated pDCs, that express both TLR7 and TLR9, are present in high numbers in synovial tissue of RA patients. However, pDCs isolated from synovial fluid display a more immature phenotype [94], that might be explained by the presence of ICs in

synovial fluid, that can inhibit DC maturation by binding to inhibitory FcR subtypes [95].

Figure 3



Model of TLR agonists as catalysts in the inflammatory processes in RA. The mechanisms by which TLRs contribute the pathogenesis of RA are multifaceted. TLR activation of DC (3) is thought to be particularly important, since this leads to T-cell activation and often initiation of Th1 responses via release of IL-12. IFNγ release by Th1 cells can subsequently activate innate immunity, for instance macrophages (5), that produce high amounts of IL-1, TNFα, IL-18 and MMPs. TLR ligation can also directly activate T-cells (4) and B-cells (2), leading to increased cytokine production and the generation of high-affinity antibodies, respectively. TLR activation of fibroblast (1) or osteoclasts (6) further results in release of pro-inflammatory cytokines, chemokines and/or MMPs (matrix metalloproteases). The mere presence of inflammatory mediators affects all the cell types present and further increases TLR expression. Collectively, the local inflammatory environment fuels the destructive processes into RA. Destruction in itself cause release of endogenous TLR agonist from damaged cartilage and bone (7) leading to a self-perpetuating loop of inflammation and contribute to the chronic character of RA.

It should be noted that activation of TLRs on other cell types besides APCs can significantly contribute to the inflammatory process, as for instance synovial fibroblasts produce a wide panel of chemokines, metalloproteinases and cytokines upon activation with TLR ligands [91, 96, 97]. It is tempting to speculate that the increased TLR expression and sensitivity for TLR ligands underlies both the pronounced state of activation of synovial tissue APCs and the high levels of pro-inflammatory

mediators found in both synovial tissue and fluid. In addition, since pro-inflammatory cytokines can increase TLR expression, these circumstances might lead to an inflammatory response with a self-perpetual character and thereby contribute to the chronicity of RA (**Fig 3**).

On another level, the use of association studies has provided some additional information on the role of TLRs in RA. The identification of the TLR4 Asp299Gly variant, that decreases responsiveness to LPS [98], enabled researchers to determine the influence of TLR4 in RA. Although earlier studies have failed to find an association between this TLR4 polymorphism and RA susceptibility [99, 100], it was recently found to be present in a statistically significant lower frequency in RA patients [101]. However, there was no association with disease severity. These findings, although they need to be confirmed by research in independent cohorts, would suggest a role for TLR4 in the initiation rather than perpetuation of RA. The contribution of (polymorphisms in) other TLRs besides TLR4 has not been subjected to similar scientific scrutiny.

In conclusion, both animal models and human studies have yielded evidence for a role of TLRs in RA but do not identify the precise contribution of TLRs to RA susceptibility and/or chronicity. Future research is warranted to unravel the exact mechanism of involvement and might lead to the potential development of novel therapeutic interventions to battle this disabling chronic disease.

NOD-LRR proteins and arthritides

The recent discovery of the clear association between mutations in NOD2 and CD, has unleashed research to investigate the potential role of NOD2 and other NOD-LRR proteins in susceptibility to a variety of arthritic conditions. Thus far, no association between mutations in NOD2 and susceptibility to RA [102, 103], psoriatic arthritis [104, 105] or ankylosing spondylitis [106] has been established and the only clear association between NOD2 and arthritis is found in BS [71, 72]. In contrast, the dramatic consequences of mutations in NALP3 in the previously mentioned auto-inflammatory disorders MWS, FCAS and NOMID opens up possibilities for an as yet unidentified role of members of this protein family in other inflammatory disorders that might seriously affect the joint. Recently, PBMC from patients with systemic onset juvenile idiopathic arthritis (SoJIA), a severe disease encompassing approximately 10% of all cases of arthritis that begin in childhood, were found to release large amounts of IL-1β upon activation [107]. IL-1RA administration to a small group of SoJIA patients resulted in complete remission in the majority of subjects, indicating the essential contribution of

IL-1β to this disease. Remarkably, the efficacy of IL-1RA in these children is in sharp contrast to that of blocking TNF [107]. A significant body of evidence from the clinic as well as animal models, has implicated that IL-1β also plays a crucial role in the pathogenesis of RA [108, 109]. However, despite the promising results of IL-1RA in SOJA patients, the clinical effects of IL1-RA in adult RA are thus far less impressive than these of TNFα blockers [110-112]. Thus far, no studies have been published that have investigated the potential link between genetic variations in NALP family members and RA susceptibility. Although a number of NALPs have been identified as inflammasome components, it is currently not known whether other NALP family members also form these inflammatory caspase-activating platforms. Furthermore, it is likely that many exogenous and endogenous NALP activators –and their potential role in immune disorders- will be discovered in years to come. It is tempting to speculate that future research in NALP biology and perhaps identification of novel mutations in NALP encoding genes will lead to new insights into the pathogenesis of RA and other arthritic conditions.

Conclusion

In recent years, TLRs and NOD-LRRs have unquestionably been identified as major regulators of innate and adaptive immunity. In addition, a vast body of data shows their involvement in the initiation and aggravation of many immune disorders. Receptor polymorphisms, recognition of self-components and defective feedback mechanisms can all contribute to the pathogenesis of disease. A greater understanding of the exact activation and regulation of these receptors and their signaling pathways could open up novel avenues for therapeutic strategies to combat auto-immune disorders.

Future perspective: TLRs as targets to battle inflammatory disorders

To date, a large body of evidence suggests the involvement of TLRs in many inflammatory disorders. They therefore appear as attractive targets for future therapies. Several strategies might be considered. One way of preventing TLR activation is blocking ligand-receptor interaction via application of specific monoclonal antibodies or TLR antagonists. These latter compounds can be ligand analogues that bind TLRs, but do not initiate signalling, or can interfere with ligand recognition by inhibiting the physical association between the ligand and accessory molecules [113, 114]. However, blocking antibodies or antagonists might not inhibit binding of all recognized structures, due to different ligand binding sites. In addition, binding sites of

nearly all (endogenous) ligands are still obscure, which severely impairs the use of such antibodies in the clinic. Furthermore, the exact TLR and TLR-associated pathways that drive the inflammatory disorders remain to be elucidated. Since TLR activation does not consistently lead to induction of immunity, but can also contribute to tolerance, this is particularly important to determine. Until then, the use of antibodies or antagonists holds the danger of potentiating the inflammatory circle, rather then restoring tolerance.

Another way to prevent TLR activation, would be to inhibit downstream signaling molecules, by using inhibitors of for example NFκB or MAPKs [115-118]. These compounds can reduce pro-inflammatory cytokine production following TLR ligation, and have been shown to alleviate symptoms in several experimental animal models of arthritis. However, it should be realized that these compounds can severely impair the induction of immune responses that play a crucial role in the prevention of infections. An alternative option is to exploit one of the many endogenous inhibitory pathways, since a deranged function of these pathways could potentially underlie the chronic character of many auto-immune diseases. Further research characterizing the exact mechanisms of immune regulation and the potential contribution of failing negative feedback to inflammatory disorders, could lead to novel therapeutic interventions aimed at restoring the delicate immune balance.

Finally, stimulation of specific TLRs can manipulate APC function, so that they gain tolerogenic capacities. Vaccination of autoimmune disease patients with these 'tolerogenic' APCs could result in attenuation of symptoms and/or restoration of the immune homeostasis. Along the same line, repetitive triggering of DCs via TLRs has been shown to abrogate their pro-inflammatory capacity, supporting the idea that the effect of TLR signaling in directing APC behaviour is heavily dependent on timing, combination and quantity of TLRs triggered. The use of TLR- activated, immune-stimulatory DCs has already shown promising results in the battle against malignancies [119, 120]. In the case of inflammatory disorders, stimulation with immunosuppressive compounds or cytokines (*e.g.* vitamin D3, dexamethasone or IL-10), in combination with TLR stimulation, might be more effective. Treatment with *ex vivo* instructed, tolerogenic DCs requires a stable DC phenotype that is not affected by the pro-inflammatory environment these cells will encounter *in vivo*.

Future research uncovering the exact mechanisms of TLR and NOD-LRR activation and their feedback mechanisms might be extremely rewarding. Once thorough understanding is achieved, development of immune intervention strategies targeting

these molecules or their signaling pathways could become powerful approaches in the battle against immune disorders.

Acknowledgments

This work was supported by NWO grants 901-10-092 and 918.66.615.

The authors would like to thank Dirk J. de Jong for helpful discussions.

Executive summary

Toll-like receptor (TLR) function and signaling

- TLRs are pattern recognition receptors that recognize PAMPs and endogenous danger signals
- TLR ligation results in activation of diversified signaling pathways and a tailored immune response
- Endogenous feedback mechanisms attenuate TLR signaling to prevent excessive inflammatory responses

TLR expression and localization

- TLRs are expressed in various cells and tissues that form the interface between internal and external milieu
- TLR localization within cells and tissues is aimed at instant recognition of pathogens while minimizing chance of response to self-components

Role of TLRs in adaptive immune responses

- Activation of different TLRs on dendritic cells regulates T-cell differentiation
- Some pathogens have evolved to avoid TLR activation or exploit TLRs to induce immune deviation

The NOD-LRR protein family

- NOD-LRR proteins are intracellular proteins with an LRR domain similar to TLRs
- NODs recognize different peptidoglycan moieties and synergize with TLRs for the production of cytokines
- Specific NOD-LRR subfamily members are components of the inflammasome, that regulates IL-1β processing

TLRs and NOD-LRRs in immune disorders

- Cooperation between TLR9 and FcγRIIa results in inflammatory responses against SLE DNA-IC
- TLRs are important contributors to the intestinal inflammation various mouse models
- TLR and NOD2 polymorphisms are associated with increased susceptibility to CD
- Mutations in NALP3 lead to a variety of auto-inflammatory disorders

The involvement of TLRs and NOD-LRRs in arthritides

- TLRs contribute to joint inflammation in various animal models of arthritis
- In humans, expression of TLRs in RA synovial tissue is increased
- In RA, TLR activation on several cell types results in production of higher levels of cytokines and chemokines
- The presence of inflammatory mediators and endogenous ligands in inflamed joints might lead to a self-perpetuating inflammatory loop, contributing to the chronic character of RA

Future perspectives

- Future research aimed at unraveling TLR and NOD-LRR activation and feedback pathways could contribute to the development of novel therapeutics to alleviate various immune disorders

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Chapter 3

Pattern recognition receptor profiling reveals high expression of RIG-I, MDA5 and PKR in human plasmacytoid dendritic cells

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Abstract

Dendritic cells (DCs) are professional antigen presenting cells that provide a link between innate and adaptive immunity. DCs reside in tissues where they constantly monitor their surroundings for signs of infection. Multiple DC-subsets exist and their activation by microorganisms occurs through binding of conserved pathogen-derived structures to so-called pattern-recognition-receptors (PRRs). Here we report a comprehensive overview of mRNA transcript levels for Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectins (CLRs) and RIG-like helicases (RLHs) in human monocyte-derived DCs (moDCs) at pro-and anti-inflammatory condititions. MoDCs display a broad array of different PRRs, including NLRs and CLRs with unknown function in human DCs. Detailed analysis of PPRs specialized in sensing RNA showed that human plasmacytoid DCs express significantly higher levels of RIG-I, MDA5 and PKR as compared to myeloid DC subsets. Moreover, cross-talk between TLR-matured pDCs and moDC leads to a state of viral resistance in moDCs characterized by enhanced RLH expression and protection against picornavirus infection.

Introduction

Dendritic cells (DCs) are crucial players in the decision process between tolerance and immunity. They not only participate in the innate immune response, but also orchestrate adaptive immunity via regulation of T-cell activation and differentiation [1, 2]. DCs are present in virtually all organs and tissues where they sample their environment for the presence of microorganisms. Different DC subsets exist that are specialized in regulation of distinct facets of the immune responses. For instance, plasmacytoid DCs are well equipped to recognize viral structures, resulting in potent type I IFN production [3]. In addition, myeloid DCs can recognize structures derived from several classes of microorganisms and are key to the induction of Th1 responses via release of IL-12 [4]. Recognition of microorganisms occurs via binding of pathogen-associated-molecular patterns, PAMPs, to so-called pattern-recognition-receptors (PRRs) [5]. The most intensively studied PRRs are the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), membrane proteins with unique roles in the orchestration of immune responses. Triggering of TLRs by PAMPs like double-stranded RNA (dsRNA) or lipopolysaccharide (LPS) activates distinct intracellular signaling pathways resulting in production of pro-and anti-inflammatory cytokines and upregulation of MHC and costimulatory molecules that collectively determine T-cell activation and differentiation [4, 6, 7]. CLRs bind various carbohydrate structures which results in antigen internalization, degradation and subsequent loading of processed peptides onto MHC molecules, thereby facilitating efficient antigen presentation. In addition, triggering of certain CLRs, like DCIR or Dectin-1, leads to direct activation of downstream signaling events that culminate in the release of cytokines or reactive oxygen species [8-10]. Importantly, cross-talk between CLRs and TLR signaling pathways results in modulation of immune responses, for instance in the case of DC-SIGN mediated enhancement of TLR-induced IL-10 production [11] or DCIR mediated inhibition of TLR9-induced type I IFN release by pDCs [12].

Much less is known about the PRRs belonging to the NOD-like receptors (NLRs) and RIG-like helicases (RLHs). Both RLHs and NLRs sense the presence of cytoplasmic PAMPs [13]. NLRs are structurally related proteins that sense a range of pathogenic structures and are associated with induction of pro-and anti-inflammatory cytokines, processing of IL-1β and apoptosis [14]. NLRs are named after the prototypical members NOD1 and NOD2 that sense different peptidoglycan motifs of both Gram positive and Gram negative bacteria [15, 16]. Activation of NODs results in NFκB activation and cytokine production [17, 18]. In addition, NODs have been reported to enhance TLR-induced cytokine responses in a synergistic fashion [19-22]. The RLHs

RIG-I and MDA5 respond to diverse RNA viruses through recognition of different RNA structures [23-25]. These PRRs share sequence similarity within a caspase recruitment domain (CARD) and a RNA helicase domain and signal to the mitochondrial adapter protein IPS-1 (also known as MAVS, VISA, or CARDIF) via CARD-CARD interactions [26]. Activation of RIG-I or MDA5 is of crucial importance for the production of type I interferons and thus the induction of innate antiviral immune responses [27]. The third member of the RLH family, LGP2, lacks a CARD domain and is thought to serve as a negative regulator [28, 29].

Not withstanding the relevance of the above mentioned findings, it is important to note that the vast majority of these studies were done in mice or cell lines, with little data available on expression or function of these molecules in primary human cells. In this study we investigated the expression levels of the four main PRR families in human monocyte-derived DCs (moDCs) under pro-or anti-inflammatory conditions. In addition, we determined the expression profiles of RNA sensors in different human DC subsets which revealed remarkably high expression of RLHs in plasmacytoid DCs compared to their myeloid counterparts. Finally, we showed that cross-talk between pDCs and moDCs induced a state of viral resistance in moDCs that was characterized by upregulation of RLHs and effective protection against viral infection.

Materials & Methods

Culture and stimulation of cells

Monocyte-derived DCs (moDCs) were generated from different donors as described previously [30]. Immature moDCs were harvested on day 6 using cold PBS and incubated with medium (RPMI-1640) alone or stimulated with purified LPS (100 ng/ml), LPS (100 ng/ml) + IFNγ (400 U/ml) or dexamethasone (10^{-6} M) + IL-10 (100 U/ml) at 37°C for a period of 4, 8 or 24 h. Alternatively, moDCs were stimulated using 100 U/ml IFNα2 (Roferon- A®, Roche) or a 1:100 dilution of cell-free supernatant of R848 activated-pDCs (collected 24 h after stimulation). Myeloid and plasmacytoid DCs were isolated using BDCA-3 beads and BDCA-4 beads (Miltenyi Biotec), respectively, according to the manufacturers' instructions.

RNA isolation

Total RNA was isolated from DC cultures using TRIZOL reagent (Invitrogen Life Technologies) according to manufacturer's instructions, with minor modifications. RNA integrity was determined by analyzing the ribosomal 28S and 18S bands on a 1% agarose gel. The reverse transcription reaction was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturers' instructions. Prior to performing qPCR, we excluded potential genomic DNA contamination by analyzing a '-RT' control for each sample in which the reverse transcriptase was replaced by DEPC treated milli-Q, after which qPCR on a single-exon gene (SOCS-1) was performed. When no signal (Ct>37) was detected using the –RT controls, the matching samples were considered appropriate for qPCR analysis. The cDNA was stored at -20°C until further use.

Quantitative PCR

Quantitative PCR (qPCR) analysis of gene expression in DCs was performed using TaqMan® Custom Arrays (Applied Biosystems) based on microfluidic card technology (for TLRs, NLRs, CLRs). Arrays were run on the ABI PRISM 7900HT Sequence Detection System and data were analyzed using SDS 2.2.2 software (Applied Biosystems). Alternatively, expression data were obtained using SYBR Green (Applied Biosystems) based qPCR according to the manufacturers' instructions (for cytoplasmic RNA sensors and when indicated in the text). Primer sequences are available upon request and were from the Primer Bank database [31]. Reactions were performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Data were analyzed using 7000 System SDS software (Applied Biosystems). Genes with a Ct-value higher than 37 were considered not expressed. ΔCt values were calculated for all individual genes in all donors, with that of porphobilinogen deaminase (PBGD, also known as hydroxymethylbilane synthase (HMBS)) as a reference. An Excel macro was used to generate a heatmap, where 1 greyscale encompasses 3 ΔCt units. White indicates highest expression, whereas black corresponds to undetectable expression.

Confocal microscopy

Monocyte-derived DCs (moDC) or plasmacytoid DCs (pDCs) were harvested, washed and allowed to adhere to poly-L-lysine coated coverslips in serum free medium for 1 h at 37°C. Cells were fixed with 1%

paraformaldehyde (PFA) and blocked in PBS with 3% BSA, 10 mM glycine and 2% human serum (blocking buffer, BB). For cell surface stainings, moDCs and pDCs were incubated using mouse-anti-human DC-SIGN (Beckman Coulter) or mouse-anti human BDCA4 (Miltenyi Biotec GmbH), respectively, or the appropriate isotype control in BB. Following incubation and washes, cells were incubated with isotype specific Alexa labeled goat-anti-mouse IgGs (Molecular Probes). For intracellular staining, cells were fixed using 1% PFA, permeabilized using 0.1% Triton-X100 in PBS and incubated with rabbit polyclonal anti-MDA5 followed by incubation with goat anti-rabbit IgG Alexa 488 (Pharmingen) in BB. Production of rabbit polyclonal anti-MDA5 was described previously. After final washes, cells were sealed using Mowiol (Merck) and analyzed by confocal laser scanning microscopy on a MRC1024 confocal microscope (BioRad, Hercules, CA) equipped with a 488 nm, 568 nm and 647 nm krypton/argon laser and a PlanApochromatic 60X/1.4 numeric aperture (NA) oil objective lens. Signals were collected sequentially to avoid bleed through and processed with Photoshop 7.0 software (Adobe Systems incorporated).

Flowcytometry

After harvest, cells were washed in ice-cold PBA (PBS containing BSA and azide) and added to a v-bottom 96-wells plate. After incubation for 30 min with PBA containing 2% HS, cells were stained using mouse-anti-human monoclonal antibodies against CD80 and CD86 (both BD Pharmingen) or the appropriate isotype controls on ice for a period of 30 min. Cells were washed twice in PBA and incubated with Alexa labeled, isotype specific goat-anti-mouse IgGs (Pharmingen) on ice for a period of 30 min. Cells were analyzed by flow cytometry on a FACSCalibur apparatus (BD Biosciences). Analysis was done using WinMDI 2.8 software.

Analysis of cytokine production

DCs (75 X 10^3/well) were plated out in triplicate in 96-wells round bottom wells plates and left untreated or stimulated as indicated for a period of 24 h at 37°C. Cell free supernatant was harvested and production of IL-6, TNFα or IL-12p70 was determined using Luminex multianalyte technology (Bio-Rad) or conventional ELISAs (Pierce-Endogen) according to manufacturers' instructions.

Western blot

Equal amounts of protein were separated by 7.5 % SDS-PAGE, electroblotted onto nitro-cellulose membranes (Bio-Rad), followed by probing with the indicated antibodies. Anti-RIG-I and anti-PKR antibodies were purchased from ProSci Incorporated and Becton Dickinson Transduction Laboratories, respectively. RIG-I, PKR and MDA5 antibodies were used in 1:1,000; 1:500 and 1:10,000 dilutions, respectively. After washes, membranes were incubated with IRDye anti-mouse or anti-rabbit IgG (1:15,000) (Li-Cor Biosciences). Imaging was done using the Odyssey System.

Infection and virus titration

Unstimulated and stimulated DCs were harvested using cold PBS, washed and infected with Echovirus 9 (Hill) at an MOI of 1 in serum free RPMI. After a 60 min incubation at 37^{o}C, cells were washed 3 times in an excess volume of PBS after which viral titers were determined at different time points post infection (p.i.) as described before [30].

Statistical analysis

The differences in the mean values of gene expression between different DC subsets were determined by two-tailed Student's *t* test. A P value of < 0.05 was considered a significant difference.

Results

PRR profiles in human immature monocyte-derived DCs

Messenger RNA (mRNA) levels of TLRs, CLRs, NLRs and RLHs in human moDCs from three different donors were determined using custom-designed low density arrays based on microfluidic card technology (see materials and methods) and conventional qPCR. As shown in **figure 1** and concomitant with their function as 'sentinels' of the body, human immature moDC were found to express a broad variety of PRRs. A relatively large difference between expression levels of distinct TLRs was observed. Expression of TLR4, TLR2 and TLR1 that recognize bacterial or fungal ligands was the highest, while TLRs responding to nucleic acids, *i.e.* TLR3, TLR7, TLR8 and TLR9 were expressed at much lower levels (on average 37-fold). TLR9, for long thought to be absent in human myeloid DCs was expressed by all 3 donors at low levels (TLR9 expression was up-to 6-fold lower than expression of TLR3, TLR7 or TLR8). High transcript levels of most CLRs were found in all three donors. As expected, Langerin (almost exclusively present in skin DCs known as Langerhans cells [32]) and CLEC2 (which functions as a platelet activating receptor [33]) were not expressed in moDCs. Very low BDCA2 levels were discerned in some donors. The expression levels of BDCA2 approached the detection limits of our low density arrays and might signify their exquisite sensitivity. The most abundant mRNA transcript levels were found for the mannose receptor (MR) that was expressed at comparable levels as the highest expressed TLR, TLR4, in all three donors. MR expression was followed by Dectin-1, DC-SIGN and DEC205, respectively. These findings thus confirm and extend earlier studies on a quantitative level by showing that all members of the TLR family and multiple CLRs are expressed by human moDCs.

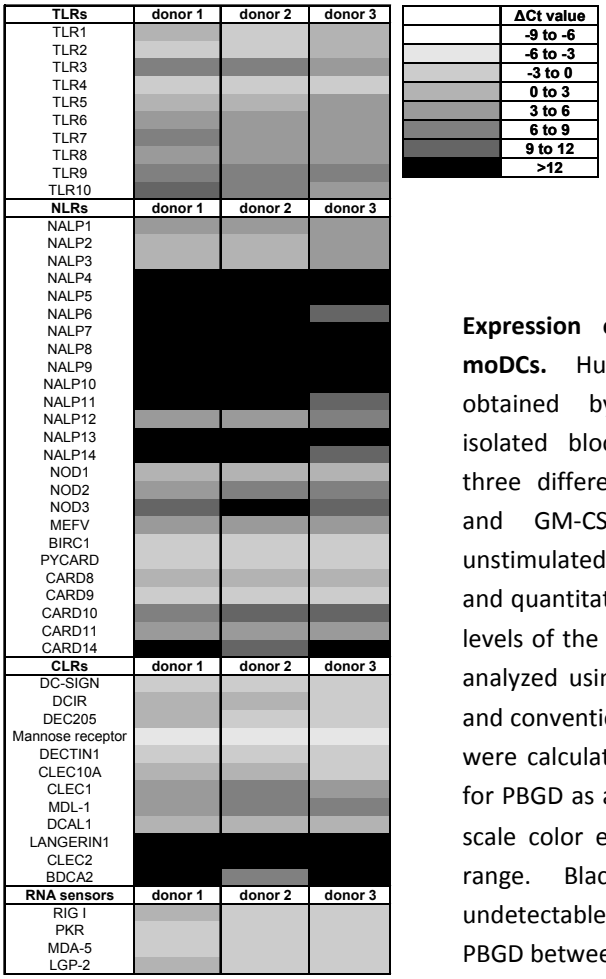
Interestingly, significant transcript levels of the RLHs RIG-I, MDA5 and LGP2 were detected in all donors, along with high levels of dsRNA activated anti-viral effector molecules like PKR. The high expression of these cytoplasmic nucleic acid sensing receptors was in sharp contrast to the relative low expression observed for their nucleic acid sensing TLR counterparts. For example, levels of RIG-I and MDA5 were found to be 25-fold and 100-fold higher than expression of TLR3 or TLR7, implying an important role for RLHs in moDC biology.

The NLR family of PRRs is still under intensive investigation and the function of the majority of the NALP subfamily is currently unknown. Although most NLRs share a molecular structure that is suggestive of PRRs, others are not true PRRs per se, but

crucial 'adapter molecules' mediating downstream signaling event upon cellular activation by PAMPs [34, 35]. Surprisingly, a large fraction (approximately 40%) of the NALP subfamily members was not detected in moDCs, which might reflect the important role that NALPs reportedly play outside the immune system [36-38]. Those NLRs with reported immune functions, *e.g.* NALP1-3, BIRC1 (NAIP5), NOD1-2, PYCARD (ASC) and CARD9 were all found to be expressed in human moDCs. Among the most highly expressed NLRs in DCs were BIRC1, CARD9 and ASC, which showed similar mRNA expression levels as TLR2/TLR4. So far, no data is present on the function of BIRC1 in DCs, while CARD9 and ASC have been shown to mediate cellular activation upon stimulation of TLRs, Dectin-1, NOD2 or other NLRs [39-42].

Collectively, these data show that human immature moDCs express a broad variety of the four main PRR families, including molecules with currently unidentified ligands or function.

Figure 1

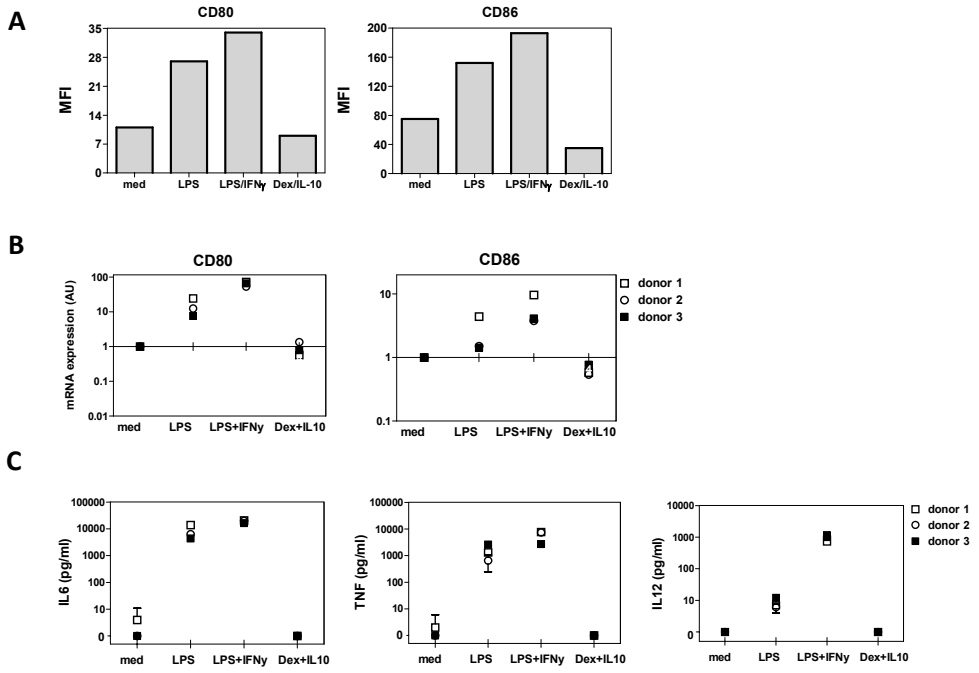


Expression of PRRs in human moDCs. Human moDCs were obtained by culturing freshly isolated blood monocytes from three different donors with IL-4 and GM-CSF. Total RNA of unstimulated cells was isolated and quantitative mRNA expression levels of the indicated genes were analyzed using low density arrays and conventional qPCR. ΔCt values were calculated with the Ct value for PBGD as a reference. One grey scale color encompasses a 3 ΔCt range. Black corresponds to undetectable mRNAs. Ct value of PBGD between 23.4-25.3

PRR expression in DCs under pro- or anti-inflammatory conditions

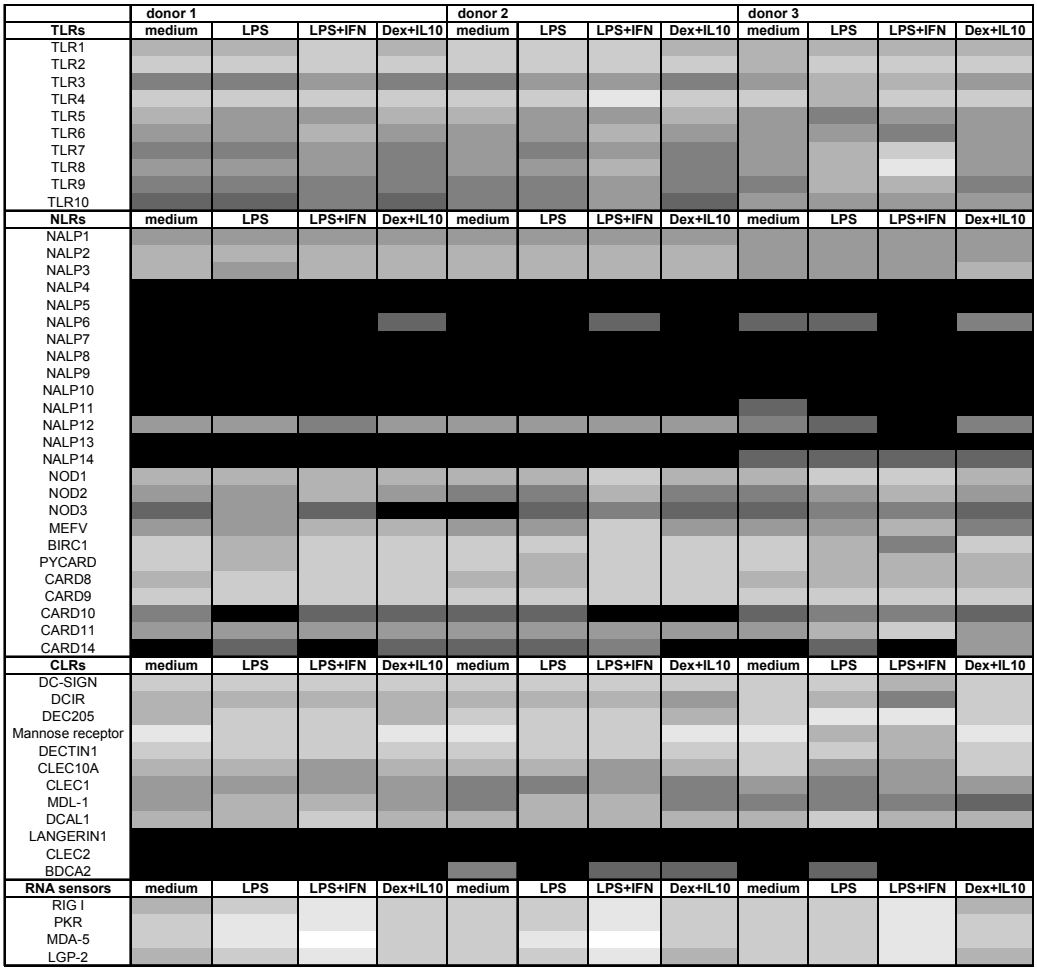
To study potential changes in PRR mRNA levels under different conditions, we stimulated DCs with LPS (100 ng/ml); LPS (100 ng/ml) + IFNγ (400 U/ml) or Dexamethasone (Dex, 10^{-6} M) + IL-10 (100 U/ml) for a period of 24 h. These stimulations were selected to represent 'maximal' pro- or anti-inflammatory conditions. As shown in **figure 2A-B**, DCs that had been treated with LPS or LPS + IFNγ displayed increased levels of CD80 and CD86, at both protein and mRNA level, while DCs that were exposed to Dexamethasone + IL-10 did not. At a functional level, DCs stimulated with LPS or LPS + IFNγ produced large amounts of the pro-inflammatory cytokines IL-6, TNFα and IL-12p70, as opposed to DCs exposed to Dex + IL-10 (**Fig. 2C**). Thus, the moDCs used for PRR mRNA expression analysis displayed the phenotype and functional properties expected upon stimulation with pro- or anti-inflammatory stimuli.

Figure 2



Phenotypical and functional properties of differently stimulated moDCs. Immature moDCs were left untreated or stimulated with purified LPS (100 ng/ml), LPS (100 ng/ml) + IFNγ (400 U/ml) or dexamethasone (10^{-6} M) + IL-10 (100 U/ml) at 37°C for a period of 24 h. Cell surface expression and mRNA expression of CD80 and CD86 were determined using flowcytometry (A) or low density arrays (B), respectively. One representative example is shown from three independent experiments using different donors. (C) Immature moDCs from three different donors were treated as described for (A) and cytokine levels in culture supernatant were determined. Graphs show mean cytokine production (+ SD) of triplicate measurements from all three donors.

Figure 3



PRR expression in DCs under pro-or anti-inflammatory conditions. Immature moDCs of three different donors were stimulated as described for figure 2. Total RNA was isolated 24 h after stimulation and quantitative mRNA expression levels of the indicated genes were analyzed using low density arrays and conventional qPCR. Analysis was performed as described for figure 1. See figure 1 for correlation grey scales / ΔCt values. Ct value of PBGD between 23.4-25.8

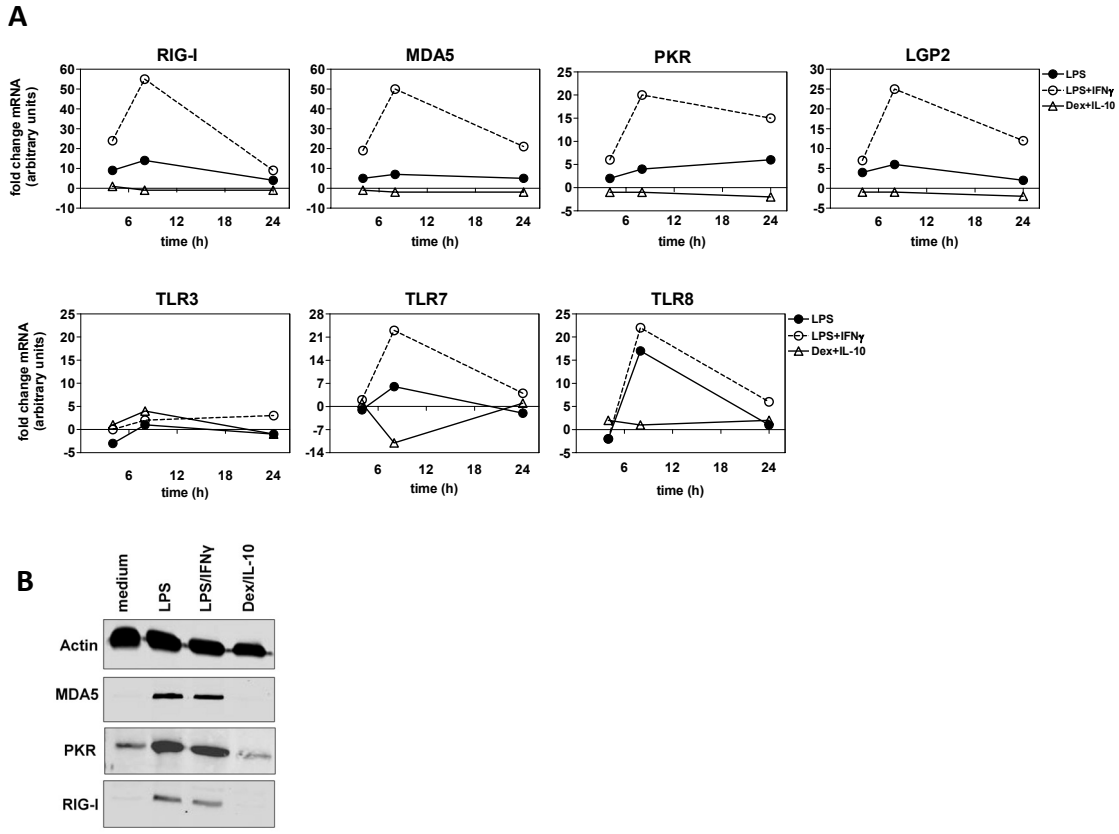
As shown in **figure 3**, some members of the TLR family showed a modest up- or downregulation following exposure to LPS/IFNγ or Dex/IL-10, respectively, and significant donor differences were observed. The NALPs that were not transcribed in DCs under 'steady-state' conditions were not induced following exposure to either pro- or anti-inflammatory stimuli (NALP6 seemed to be subject to some variation, but still remained at very low levels). We observed higher NOD2 expression (11-32-fold increase) following exposure to LPS/IFNγ, confirming in human DCs the effect of IFNγ on NOD2 expression in human intestinal epithelial cells [43]. Within the CLR family, the mannose receptor (MR) and CLEC10A (also known as HML(-2)), reported to bind

densely glycosylated O-linked glycopeptides [44], were downregulated under maximal pro-inflammatory conditions (7-28 fold for MR and 10-240-fold for CLEC10A). Although levels of other CLRs, like DC-SIGN, were also downregulated upon proinflammatory stimulation, these values mostly remained within the 3 ΔCt cut-off point used to delineate different expression intensities in the table (see materials and methods section). Interestingly, differences in expression levels were most apparent within the family of cytoplasmic viral RNA sensors. Expression levels of the RLHs RIG-I and MDA5 as well as effector molecules like PKR were significantly upregulated (on average 14-17 fold) following stimulation with LPS/IFNγ, suggesting that exposure to these proinflammatory stimuli can affect the responsiveness of DCs to viral pathogens.

Dynamics of PRR expression upon DC stimulation

Next, we investigated whether PRR mRNA expression profiles would shift in DCs cultured under inflammatory or tolerogenic conditions for other time points than 24 h. Therefore, we analyzed kinetics of PRR expression following stimulation of DCs, focusing on PRRs involved in recognition of viral pathogens, *e.g.* TLR3, TLR7, TLR8 and the cytoplasmic viral RNA sensors. This showed that upregulation of TLRs and RLHs was more pronounced than initially observed at our 24 h time point and peaked approximately 8 h after exposure to pro-inflammatory stimuli. The expression of most genes had declined again 24 h following stimulation, some almost equaling baseline levels (**Fig. 4A**). Dex/IL-10 had little effect on TLR or RLH transcript levels at either early or late time points. The combination of LPS+IFNγ caused a more pronounced upregulation of PRRs than LPS alone. TLR3 expression showed surprisingly little modulation in time compared to the other TLRs and RLHs studied. To determine whether the profound changes in PRR mRNA expression in stimulated DCs were also observed at the protein level, western blot (WB) analysis was performed on a selection of candidates using antibodies specific for RIG-I, MDA5 and PKR. The WB results corroborated our findings obtained via qPCR and low density arrays, by showing increased protein levels of these molecules in DCs following 24 h treatment with LPS and LPS+IFNγ, but not Dex/IL-10 treated DCs (**Fig. 4B**). Thus, moDCs rapidly upregulate expression of PRRs responding to viral RNA at the mRNA and protein level upon exposure to PAMPs and pro-inflammatory stimuli that might result in an increased capacity to respond to microorganisms.

Figure 4



Kinetics of PRR expression in DCs following pro-or anti-inflammatory stimulation. (A) Immature moDCs of three different donors were stimulated as described for figure 2 and total RNA was isolated 4, 8 or 24 h after stimulation. Quantitative mRNA expression levels were analyzed using conventional qPCR. PBGD was used as reference gene. One representative example of three independent experiments using different donors is shown. (B) DCs were stimulated as described for figure 2 and protein levels of RIG-I, MDA5, PKR and actin were analyzed using western blot 24 h after stimulation. Shown is a representative example from two independent experiment using different donors.

Expression of RNA sensors in blood myeloid DCs, plasmacytoid DCs and moDCs

MoDCs are frequently used as an alternative for the rare populations of myeloid (m)DCs found *in vivo*. Although many of the phenotypical and functional characteristics are shared between these two cell types [45], differences do exist [46]. To further study the RLH-family of RNA sensors in human DC subsets, we determined their expression in freshly isolated mDCs and plasmacytoid (p)DCs, the latter playing a crucial role in antiviral immunity. The expression of TLR3, TLR7 and TLR8 that are also known to be involved in viral nucleic acid recognition were analyzed in parallel.

Quantitative analysis showed that TLR8 levels were approximately 13-fold higher than levels of TLR3 or TLR7 in moDCs, while differences in expression of these TLRs in freshly isolated mDCs were less pronounced (**Fig. 5A**). In contrast, pDCs were found to express approximately 100-fold higher levels of TLR7 as compared to TLR8 and as much as 500 times more TLR7 than TLR3. Accordingly, pDCs show only a modest response to TLR8 agonists and don't respond to poly(I:C) (data not shown and references [4, 47]). In addition, highly divergent TLR expression profiles were detected between the different DC subsets analyzed. The levels of TLR3 were highest in mDCs compared to moDCs or pDCs that showed 18-fold and 32-fold lower expression, respectively. The observed differences in expression of TLR7 were even more dramatic; pDCs showed 40- and 221-fold higher TLR7 levels as compared to moDC and mDC (**Fig. 5B**). No data is currently present on expression of RLHs and related molecules in human mDCs or pDCs. We found that all DC subsets expressed significant mRNA levels of RIG-I, MDA5, PKR and LGP2 (**Fig. 5C**). Of these genes, LGP2 expression was the lowest in all DC-types analyzed, which might be associated with its proposed function as negative regulator of MDA5 and RIG-I responses [28]. For both myeloid DC subsets only a relatively modest difference was observed in expression levels of RIG-I and MDA5. In contrast, pDCs expressed considerably higher levels of MDA5 compared to RIG-I (**Fig. 5C**). Interestingly, pDCs expressed both RIG-I and PKR at significantly higher levels than moDCs or mDCs (**Fig. 5D**). The most striking difference was observed for expression of MDA5 mRNA, which was expressed in pDCs at 11-fold and 23-fold higher levels than in moDCs or mDCs, respectively. The abundance of MDA5 in pDCs was confirmed at the protein level by confocal microscopy (**Fig. 5E**). The distribution pattern of MDA5 protein in pDCs was reminiscent of its proposed cytoplasmic localization. The high expression of not only TLR7, but also cytoplasmic RNA sensors and antiviral effector molecules in pDCs provides additional evidence for specialization between DC subsets and the crucial role of pDCs in antiviral immunity. This was further substantiated by the high constitutive mRNA levels of interferon regulatory factor 7 (IRF-7), a key transcription factor in the innate immune response against viral infections through activation of type I IFN genes and other interferon-stimulated genes (ISGs), which was 143 and 24 times higher than in moDCs or mDCs, respectively (**Fig. 5F**).

Plasmacytoid DC mediated anti-viral immunity in moDCs

The high expression of PRRs involved in recognition of viral RNA, including RLHs and TLRs in pDCs, emphasizes their crucial role in the antiviral response. We investigated if cross-talk between pDCs and moDCs could upregulate the expression of cytoplasmic

RNA sensors to create a state of antiviral resistance in the latter. Therefore, the levels of RIG-I, MDA5 and PKR were analyzed in moDCs that were exposed to supernatant of pDCs activated with TLR7/8 ligand R848.

Figure 5

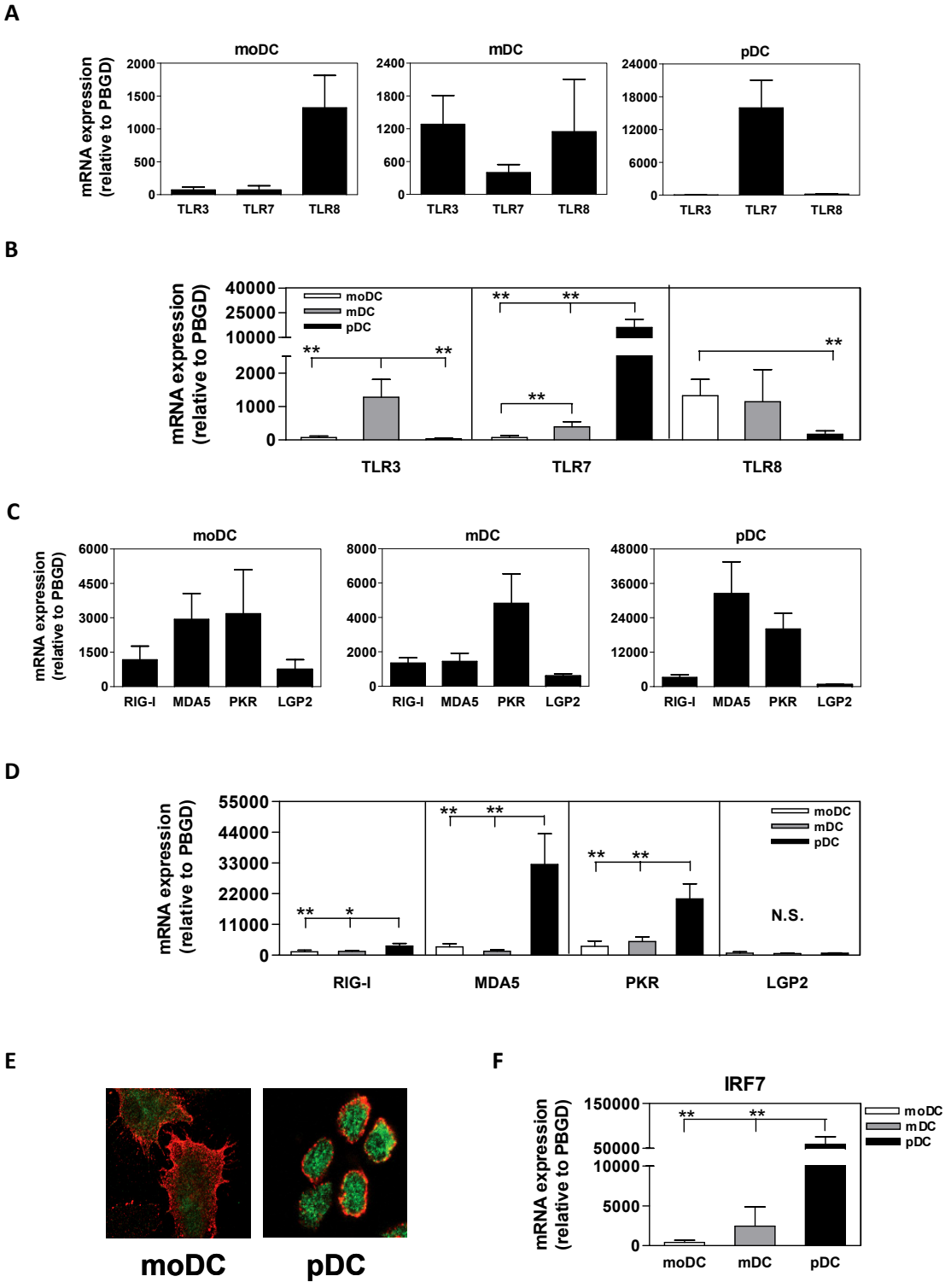
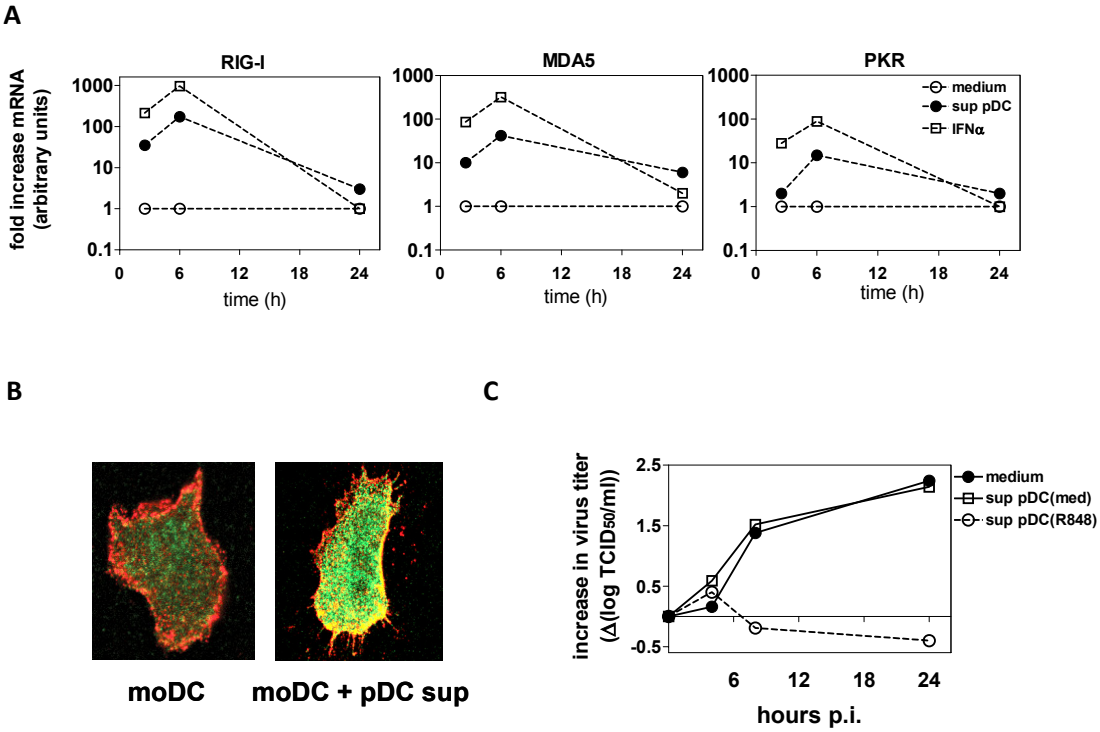


Figure 5. Human DC subsets express different profiles of viral nucleic acid receptors (legend figure 5). (A) Mean expression (+ SD) of TLR3, TLR7 and TLR8 in human moDCs, freshly isolated mDCs and freshly isolated pDCs. RNA was isolated from unstimulated cells and TLR mRNA transcript levels determined using conventional qPCR. Expression levels relative to PBGD. (B) Comparison of expression levels of TLR3, TLR7 and TLR8 in human DC subsets. Expression levels were determined using qPCR as described for (A). ** P<0.01. (C) Expression levels of RIG-I, MDA5, PKR and LGP2 in resting human DC subsets were determined as described for (A). Shown is mean expression + SD. (D) Comparison of expression levels of RIG-I, MDA5, PKR and LGP2 in different DC subsets. Quantitative mRNA expression was determined as described for (A) and mean expression (+ SD) is given. * P<0.05; ** P<0.01. (E) Confocal laser scanning microscopy analysis of MDA5 expression in human moDCs and pDCs. Unstimulated cells were stained using antibodies against DC-SIGN and MDA5 (for moDCs) or BDCA2 and MDA5 (for pDCs) followed by incubation with Alexa labeled secondary antibodies. Green indicates MDA5 expression, red indicates DC-SIGN or BDCA2 in moDCs and pDCs, respectively. A representative example of three independent experiments using different donors is shown. (F) IRF7 expression in human DC subsets was determined using qPCR as described for (A). Shown is mean expression + SD. ** P<0.01. Mean quantitative gene expression + SD from 6 moDCs, 3 pDCs and 3 mDCs is displayed (for figures A-D and F).

We have shown before that R848 does not induce upregulation of cytoplasmic viral sensors in moDCs [48]. In contrast, pDC supernatant that was as much as 100 X diluted still triggered a profound increase in RIG-I, MDA5 and PKR transcript levels in moDCs (**Fig. 6A**). Exposure of moDCs to recombinant IFNα mimicked the effect of pDC supernatant, suggesting that the observed mRNA upregulation is at least partly mediated via pDC derived type I IFN (**Fig. 6A**). The increased mRNA levels of MDA5 in moDCs were confirmed at the protein level by confocal analysis (**Fig. 6B**). Interestingly, MDA5 has been reported to play a crucial role in the responses to the picornavirus EMCV in mouse DCs [24] and we recently showed that echoviruses, single-stranded RNA viruses belonging to the picornavirus family, can efficiently infect in human moDCs[30]. To study whether upregulation of MDA5 (and other viral sensors) was associated with decreased susceptibility for infection, moDCs were exposed to pDC supernatant and infected with EV9. EV9 titers increased more than two logs in untreated moDCs or moDCs exposed to supernatant of unstimulated pDCs. In contrast, treatment with supernatant of R848-activated pDCs completely inhibited EV9 replication in human moDCs (**Fig. 6C**).

In conclusion, these data suggest that pDC activation can increase the resistance against viral infection in DCs from the myeloid lineage, which underlines the importance of cross-talk between DC subsets in modification of the biological response to pathogens.

Figure 6



Human pDC induce a state of antiviral resistance in moDCs that inhibits infection with EV9 (A) Human moDCs were exposed to cell free supernatants of pDCs (in a 1:100 dilution in RPMI 1640) that had been stimulated with R848 (4 ug/ml) for a period of 24 h. Alternatively, moDCs were stimulated using 100 U/ml IFNα2. Total RNA was isolated at the indicated time points following stimulation and mRNA transcript levels determined using qPCR with expression of PBGD as a reference. Expression is given relative to unstimulated cells (medium). (B) Confocal laser scanning microscopy analysis of MDA5 expression in human moDCs that were left untreated or exposed to supernatant of pDC activated with R848 as described for (A). Cells were stained using antibodies against DC-SIGN and MDA5 followed by incubation with Alexa labeled secondary antibodies. Green indicates MDA5 expression; red indicates expression of DC-SIGN. (C) Human moDCs were exposed to cell free supernatants of differently stimulated pDCs for a period of 24 h and subsequently harvested, washed and exposed to EV9 at an MOI of 1 in serum free medium for 1 h at 37°C. After washes to remove unbound virus, cells were plated out and viral titers determined at several time points after infection. Shown is a representative example of two independent experiments using different donors.

Discussion

DCs act as sentinels of the body. They are present in virtually all tissues and constantly monitor their surroundings for signs of infection. To recognize microorganisms, DCs express a vast array of PRRs. Although a large body of information is available regarding the expression and function of TLRs and CLRs in DCs, data on expression and function of many different NLR and RLH family members in human DCs is virtually absent. Therefore, we set out to determine the quantitative expression levels of the four major PRR families in human DCs at different experimental conditions, with a special focus on PRRs involved in recognition of viral RNA.

Human moDCs were found to express transcripts for all TLR family members, including TLR9. The mean expression levels of TLRs responding to nucleic acids were considerably lower than those responding to bacterial or fungal pathogens. This could represent a mechanism to prevent unwanted responses to host nucleic acids that have been associated with autoimmune diseases like systemic lupus erythematosus [49, 50]. In this respect, it is interesting to note that increased expression levels of TLR7 are directly linked to acceleration of autoimmunity in several mouse models of lupus [51].

As expected, many CLRs family members were found to be expressed by moDCs. For a number of CLRs, like DCAL-1 and CLEC1, no ligand or function has been identified. Experiments using a DCAL-1 fusion protein have suggested that it may act as a T-cell costimulatory molecule, which skews T-cell differentiation towards a Th2 response [52]. Further investigation into the role of these poorly characterized CLRs in DC biology might provide more insight into diseases like asthma or allergies, which might be linked to DC-mediated derailment of Th2 responses [53, 54]. In addition, more detailed knowledge regarding the expression level and function of these CLRs might be brought to use in DC antigen-targeting strategies.

While all TLRs and most of the CLRs analyzed were expressed, human moDCs displayed an NLR expression profile in which a significant proportion of the NALP subfamily members were absent. In line with this observation, many of the non-expressed NALPs appear to play a role outside the immune system; NALP10 is expressed constitutively in cerebellar neurons [36] and NALP5 (also known as MATER, *maternal gene that embryos require*) is expressed in oocytes and functions in embryonic development [37, 38]. Although certain NLRs expressed in human moDCs are reportedly endowed with immune functions (*e.g.* NALP1, NALP2, NALP3, NAIP), their role in DC biology in humans is currently unknown. Highest expression was found for the NLR BIRC1, which

is involved in the response to *Legionella pneumophila* [55, 56]. It will be interesting to determine the potential contribution of BIRC1 in the response of DCs to this important human pathogen. Also CARD9 showed high constitutive expression in moDCs. This NLR is not a true PRR per se, but an 'adaptor molecule' that has recently been identified as a crucial signaling component in myeloid cells following activation of Dectin-1, NOD2 and selective TLRs [34, 39-41]. In contrast, expression of CARMA1 (CARD11), an NLR that is closely related to CARD9 was 50-fold lower. CARMA-1 is an adaptor protein in lymphocytes that links triggering of T-cell receptors and B-cell receptors to activation of NFκB [57, 58]. These findings underscore that analysis of PRRs and their signalling components might provide important clues regarding the respective function of different immune cells and further substantiate the concept of cross-talk between different PRRs.

Expression of PRRs can be altered under pro- or anti-inflammatory conditions [43, 59]. Only modest differences in PRR expression levels were observed 24 h following exposure of DCs to pro- or anti-inflammatory stimuli. The group of cytoplasmic viral RNA sensors showed the most significant upregulation following stimulation with LPS + IFNγ. Interestingly, detailed analysis of the kinetics of mRNA expression showed that the upregulation of these molecules at early time points following stimulation was even more pronounced, indicating the importance of including multiple time points during mRNA expression analysis. The fact that not only RLHs, but also TLRs responding to viral RNA were rapidly upregulated following exposure to LPS + IFNγ suggests that DCs entering a site with ongoing infection can rapidly increase their capacity to induce both innate and adaptive responses against different classes of microorganisms. The upregulation of negative feedback molecules, like members of the *suppressor of cytokine signaling* (SOCS) family at later time points following exposure to proinflammatory stimuli (data not shown) likely serves to prevent excessive inflammation.

Myeloid DCs and pDCs have been shown to display distinct TLR profiles that enable them to respond to different microbial structures [4]. We here confirmed these data on a quantitative level and showed that expression of TLR7 in pDCs vastly exceeded that of TLR3 and TLR8. This is in accordance with the findings that pDCs respond strongly to synthetic TLR7-, but not TLR8-specific ligands [47] and are unresponsive to the synthetic dsRNA mimic poly(I:C) [4]. In contrast, moDCs expressed much higher levels of TLR8 compared to TLR3 and TLR7. The highly divergent expression profile of TLR7 and TLR8 in human pDCs and myloid DC subsets likely reflects their ability to induce specific responses following recognition of different virus structures.

Virtually no data is currently present on RIG-I and MDA5 in human DCs and our study provides important new insights into the expression of these RLHs in different human DC subsets. We found that all human DC subsets analyzed expressed considerable levels of RLHs and the antiviral effector molecule PKR. A recent mouse study has suggested that RIG-I is crucial for the antiviral response of myeloid DCs following exposure to the RNA virus NDV (*Newcastle disease virus*). In contrast, in pDCs RIG-I was shown to be dispensable and the antiviral responses against NDV were dependent on TLRs [60]. Interestingly, our finding that RIG-I mRNA levels in human pDCs are significantly higher than the levels found in moDCs and mDCs suggests that in the human setting RIG-I could play a pivotal role in the response of pDCs to RNA viruses. Even more pronounced were the expression levels of MDA5 in human pDCs that were remarkably higher than those found in moDCs or mDCs. MDA5 has recently been shown to mediate type I IFN responses following exposure of mouse myeloid DCs to poly(I:C) and the picornavirus EMCV (*encephalomyocarditisvirus*) [24]. Whether the prominent expression of MDA5 signifies a crucial role of this molecule in the response of pDCs to picornaviruses remains to be determined. It could be envisioned that the high constitutive level of TLR7, RLHs and the effector molecule PKR in pDCs might not only mediate the robust type I IFN responses of pDCs to viral pathogens but could also protect these cells from becoming infected themselves. Finally, we showed that pDCs can confer a state of viral resistance to other DC subsets. The expression of RLHs and antiviral effector molecules in moDCs was dramatically upregulated following exposure to supernatant of activated pDCs and in addition caused complete protection of moDCs against infection with the picornavirus EV9.

In conclusion, we showed that human moDCs express a broad variety of PRRs, some with a currently unknown function in DC biology. Detailed time point analysis showed that exposure of moDCs to LPS + INFγ caused a prominent but transit increase in the mRNA expression of RLHs and TLRs responding to viral RNA. Expression profiling of PRRs involved in the antiviral response demonstrated that pDCs express significantly higher levels of the RLH family members RIG-I, MDA5 as well as PKR and IRF-7 compared to myeloid DCs. The profound expression of these antiviral genes in human pDCs provides a possible explanation as to how these cells survive and function in an environment with high virus burden. Finally, cross-talk between pDCs and moDCs can enhance the expression of RIG-I, MDA5 and PKR in moDCs and create a state of antiviral resistance that protects these cells against viral infection. Further investigation into the novel findings regarding RLHs in human DCs reported here will contribute importantly to our insight into DC-pathogen interaction.

Acknowledgments

The authors would like to thank Daniel Benitez-Ribas and Jolanda de Vries (department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Medical Centre, Nijmegen, The Netherlands) for help with the pDC experiments and Paul Fisher (Departments of Pathology, Neurosurgery and Urology, Columbia University, College of Physicians and Surgeons, New York, USA) for providing us with anti-MDA5 antibody. This work was supported by grants from the Netherlands Diabetes Foundation (DFN 2001.00.047) to G.J.A and The Netherlands Organization for Scientific Research (NWO-VIDI-917.46.305) to F.J.M.v.K. and (NWO-912-02-034) to G.J.A.

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Chapter 4

Impaired dendritic cell function in Crohn's disease patients with NOD2 3020insC mutation

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Journal of Leukocyte Biology (2006) 79: 860-866

Abstract

The NOD2 3020insC mutation (NOD2fs) increases susceptibility to Crohn's disease (CD), but the mechanism remains controversial. Both loss-of-function and gain-of-function phenotypes have been described as a result of NOD2fs. Here, we show that dendritic cells (DC) derived from CD patients homozygous for this mutation respond normally to purified TLR ligands but fail to up-regulate the costimulatory molecules CD80 and CD86 in response to the NOD2 ligand muramyl dipeptide (MDP). Moreover, they lack MDP-induced enhancement of TLR-mediated TNFα, IL-12 and IL-10 production that is observed in control DC with intact NOD2. These data indicate that the 3020insC NOD2 mutation results in a loss-of-function phenotype in human myeloid DC and imply decreased immune regulation by IL-10 as a possible mechanism for this mutation in CD.

Introduction

Nucleotide oligomerization domain 2 (NOD2; CARD15) is the first gene identified within the IBD1 locus and different NOD2 mutations are found to be associated with Crohn's disease (CD). Although mutations in NOD2 do not inevitably lead to the development of CD, individuals that are homozygous for the 3020insC (NOD2fs) mutation show a dramatically (up to 38-fold) increased susceptibility [1, 2]. Thus, homozygous NOD2fs individuals constitute a unique subset of patients to study the contribution of this mutation to the pathogenesis of CD.

NOD2 is a cytoplasmic protein that binds muramyl dipeptide (MDP), a motif common to peptidoglycan (PGN) of both $Gram^+$ and $Gram^-$ bacteria, resulting in NFκB activation and cytokine induction [3]. Furthermore, NOD2 activation has been suggested to affect Toll Like Receptor (TLR)-mediated responses [4, 5].

The mechanism by which NOD2fs contributes to CD remains largely unresolved. Recent data from several NOD2 mouse models have yielded surprisingly different conclusions. TLR2-induced IL-12 production by myeloid cells was found to be decreased after co-stimulation with MDP, an effect absent in $NOD2^{-/-}$ mice, implying that NOD2 is a negative regulator of TLR2 mediated Th1 responses [6]. In line with this gain-of-function phenotype of KO mice, NOD2fs knock-in mice showed enhanced NFκB activity and IL1β secretion in response to MDP as well as increased susceptibility to bacterial-induced intestinal inflammation [7]. However, another mouse study demonstrated that NOD2 activation in wild-type mice enhanced TLR-mediated cytokine production by bone marrow derived macrophages, while NOD2-deficient animals had decreased cryptdin expression in terminal ileum cells and impaired anti-bacterial responses after challenge with *L. monocytogenes* [8], thus favoring a loss-of-function phenotype. Few studies have yet focused on the effect of NOD2fs in human DC, that are the only antigen presenting cells capable of activating naive T-cells and the induction of both immunity and tolerance [9]. Also in the gut, DC are crucial for the induction of tolerance to the resident intestinal flora [10-12]. We therefore aimed to determine the consequence of NOD2fs for the responsiveness of DC to MDP, focusing on maturation and cytokine production. We specifically studied IL-12, TNFα and IL-10, considering their importance in affecting inflammatory processes in general and the pathogenesis of CD in particular [13-16].

Materials & Methods

Patients and genotyping of NOD2 variants

Blood was collected from 150 CD patients and 10 healthy volunteers. PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50-µl reaction volumes containing 100–200 ng of genomic DNA. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI PRISM 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems). Four CD patients were found to be homozygous for the NOD2fs mutation, and were selected for further studies. As control groups, 4 patients with CD bearing the wild-type allele, and 5 healthy volunteers homozygous for the wild-type NOD2 allele were included. Approval from the local regulatory committee was obtained.

Isolation of mononuclear cells and generation of monocyte derived DC

After informed consent, 80 ml venous blood was drawn from the cubital vein of patients and healthy volunteers into 10-ml EDTA tubes (Monoject; 's-Hertogenbosch). Total blood was diluted 1/1 using PBS containing 0.45% sodium citrate (dilution solution) and mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (1.077 g/ml; Axis Shield PoC AS) according to manufacturer's instructions. Cells were washed once in dilution solution at 1800 rpm for 8 minutes to remove Lymphoprep remains and further washed (1500 rpm/5 minutes) using ice cold dilution solution containing 1% FCS until clear supernatant was obtained. After counting, cells were resuspended in RPMI containing 2% HS at a density of 10-12.5x10^6 cells/ml and plated in T25 or T75 culture flasks (Costar) at 37°C for a period of 1 hour. Non-adherent cells were removed by washing 3x with PBS and adherent cells were cultured in RPMI 1640 supplemented with 100 U/ml antibiotic-antimycotic (Invitrogen), 10 mM L-glutamine and 10% FCS. To generate monocyte-derived DC, 400 U/ml IL-4 and 800 U/ml GM-CSF (Schering-Plough) was added to the culture medium. Medium was refreshed and complete cytokines added at day 3. On day 6, immature DCs were harvested using cold PBS and cells were stimulated as indicated below. An average yield of 2.3x10^6 (SD 0.7x10^6) immature DC was obtained per donor.

Stimulation of DC

For cytokine analysis: immature moDC (50x10^3) in 100 µl medium were added to round-bottom 96-wells plates (Costar) with 50 µl culture medium (negative control) or 50 µl medium containing the various stimuli; purified LPS (*Escherichia coli*, 100 ng/ml), synthetic Pam3Cys (10 µg/ml, EMC Microcollections), synthetic poly(I:C) (20 µg/ml, Sigma-Aldrich) in the presence or absence of MDP (5 µg/ml; Sigma-Aldrich), IFNγ (400 U/ml) or a combination of both for a period of 24 hours. For flowcytometry; immature moDC (0.6x10^6) in a 1.5 ml volume were added to 12-well plates (Costar) and incubated with 20 µg/ml poly(I:C) or 10 µg/ml Pam3Cys for a period of 24 hours after which the expression of costimulatory molecules was analyzed using flow cytometry.

Flowcytometric analysis of costimulatory molecule expression on DC

After 24 hour incubation with indicated TLR-ligands, cells were harvested and washed using ice-cold PBA (PBS containing BSA and azide). Cells (50x10^3 cells/well) were added to a v-bottom 96-wells plate

(Costar) and stained using CD80 or CD86-specific mouse-anti-human IgG1 antibodies or the appropriate isotype control (BD, Pharmingen) on ice for a period of 30 minutes. Cells were washed twice in ice-cold PBA and incubated with phycoerythrin (PE) labeled goat-anti-mouse Ig (BD Pharmingen) on ice for a period of 30 minutes. After double washings, cells were incubated in 200 µl PBA and the expression of CD80 and CD86 analyzed via flowcytometry (FACSCalibur, BD). Analysis was done using WinMDI 2.8 software.

Cytokine measurements

After 24 hour stimulation, supernatants were harvested and stored at -80°C until cytokine analysis was performed. IL-12 concentrations in supernatants were measured using an IL-12p70 specific sandwich ELISA (Pierce-Endogen). TNFα and IL-10 levels were determined using commercially available kits (Bio-Rad Laboratories) according to the manufacturer's instructions. Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistical analysis

Differences in the increase in MFI of CD80 and CD86 after 24-hour stimulation with TLR ligands or MDP between individuals homozygous for NOD2fs (n=4) and those with intact NOD2 (4 CD patients and 5 healthy controls) were analyzed using the Student's t-test. Stimulation experiments for cytokine analysis were performed in duplo. Differences in cytokine production between cells from patients homozygous for the NOD2fs polymorphism (n=4) and cells from individuals bearing the wild-type allele (4 CD patients, 5 healthy controls) were analyzed using the Student's t-test.

Results

TLR activation of NOD2fs DC leads to normal maturation and cytokine production

To determine whether the NOD2fs mutation affects TLR-mediated DC activation, we analyzed DC maturation (a hallmark of activation) via the expression of CD80 and CD86 on monocyte-derived DC from (I) healthy controls, (II) CD patients with intact NOD2 and (III) CD patients homozygous for NOD2fs. For patient characteristics, see **Table 1**.

Table 1

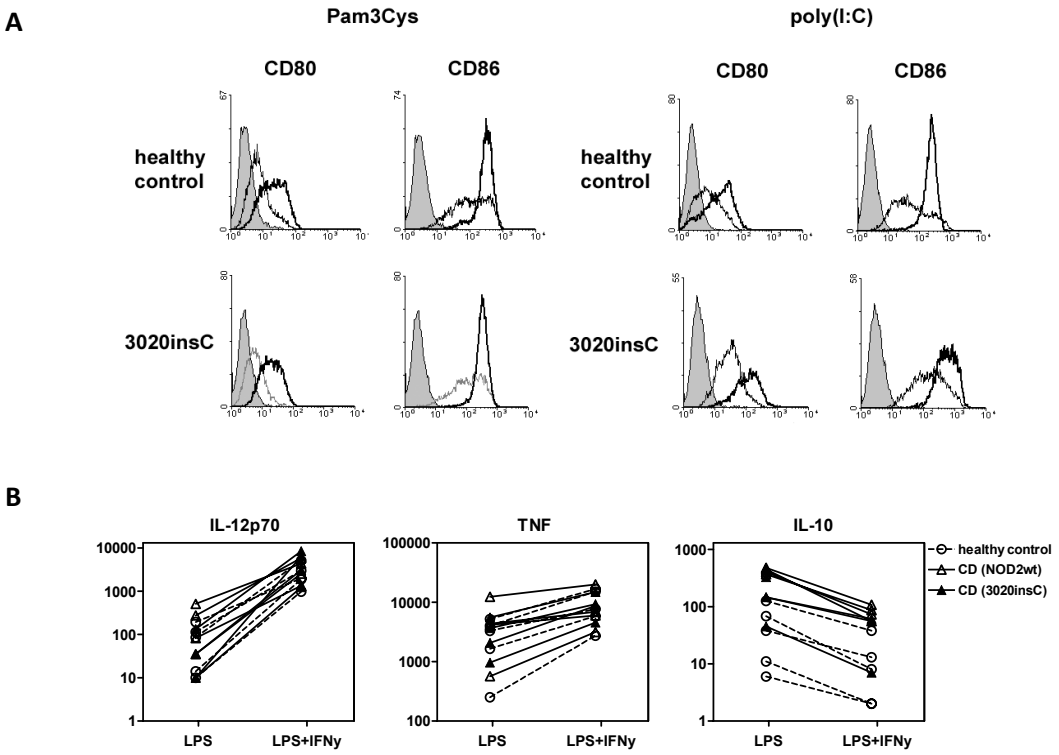
donor	Age	Sex	Disease duration (y)	Fistulae	Resections	Medication
Healthy control	26	M	*	No	No	-
Healthy control	36	F	*	No	No	-
Healthy control	35	F	*	No	No	-
Healthy control	36	M	*	No	No	-
Healthy control	38	M	*	No	No	-
CD (NOD2wt)	56	M	28	No	No	#
CD (NOD2wt)	53	M	34	Yes	No	†, §
CD (NOD2wt)	48	M	29	Yes	No	†, §
CD (NOD2wt)	60	M	11	Yes	Yes	‡
CD(NOD2fs)	61	F	27	Yes	Yes	#, †, §
CD(NOD2fs)	57	M	38	No	Yes	#
CD(NOD2fs)	41	M	19	Yes	Yes	†
CD(NOD2fs)	54	F	36	yes	Yes	§

Overview of general patient characteristics, relevant clinical information and prescribed medication at the time of study. None of the patients included received prednisolone during the study. Three patients (two NOD2fs CD patients and one NOD2+/+ CD patient) had been treated with Infliximab, the last infusion being more then 6 months prior to study. Taking into account the half-life of 9 days, no relevant residual Infliximab activity could be expected. † azathioprine; ‡ 6-mercaptopurine; # budesonide; § mesaline.

DC were stimulated with synthetic or highly purified TLR ligands to avoid possible effects of contamination with enterotoxins. As shown in **figure 1A**, irrespective of their NOD2 genotype, DC stimulated with the synthetic TLR1/2 ligand Pam3Cys (PAM) or TLR3 ligand poly(I:C) responded with similar upregulation of the DC maturation markers CD80 and CD86. The minor differences observed in absolute expression levels between different individuals fall within the normal donor variation. We note that a similar upregulation of those molecules was observed when using (healthy control) DC generated with human serum instead of FCS (data not shown). To assess cytokine responses of DC from NOD2fs patients in response to TLR activation, we measured IL-

12, TNFα and IL-10 after stimulation with TLR-ligands in the presence or absence of IFNγ. IFNγ is known to be present in increased amounts in the gut of CD patients and can enhance TLR and NOD2 responses. No significant difference in cytokine induction by LPS alone (IL12p70 P=0.31, TNFα P=0.32, IL-10 P=0.18) or in combination with IFNγ (IL12p70=0.23, TNFα P=0.44, IL-10 P=0.16) between NOD2fs DC and control DC was observed (**Fig. 1 B**).

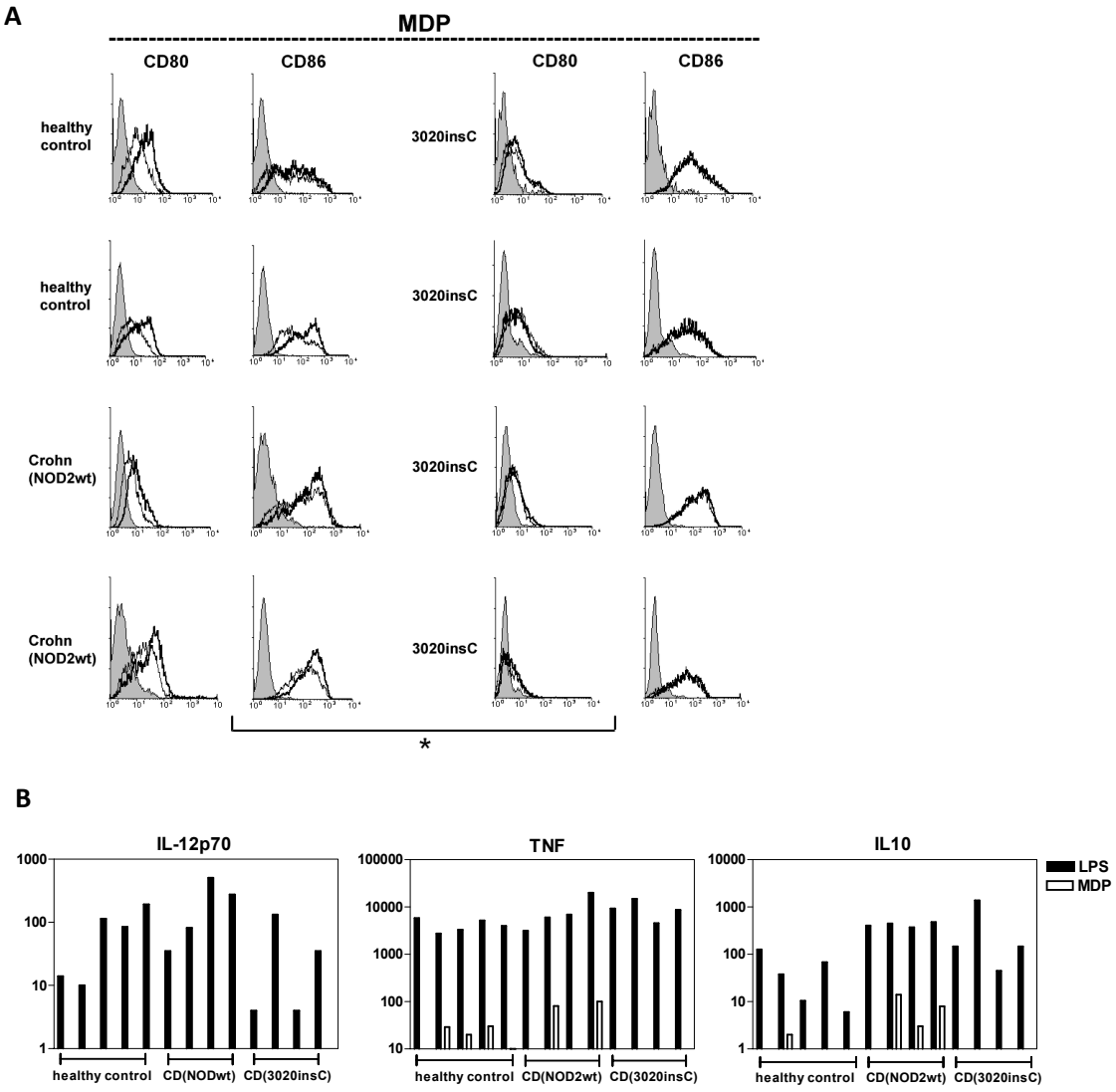
Figure 1



TLR activation is not affected in NOD2fs DC (A) DC were generated by culturing monocytes in the presence of IL-4 and GM-CSF for a period of 6 days. DC from healthy controls or NOD2fs patients were stimulated with synthetic TLR ligands Pam3Cys-SKKKK (PAM, 10 µg/ml) or polyinosinic-polycytidylic-acid sodium salt (poly(I:C), 20 µg/ml) for 24 hours. CD80 and CD86 expression on the cell surface was analyzed by flowcytometry. Maturation responses shown are representative of 6 healthy controls and 4 NOD2fs patients. Medium control and indicated TLR stimulation represented by thin and thick lines, respectively. Filled histogram indicates isotype-matched control mAb. (B) Production of IL-12, TNFα and IL-10 by DC from healthy controls (n=5), $NOD2^{+/+}$ CD patients (n=4) or homozygous NOD2fs CD patients (n=4). Cells were stimulated with LPS (*Escherichia coli*, 100 ng/ml) alone, or LPS in combination with 400 U/ml IFNγ for a period of 24 hours after which cytokine concentrations in culture supernatants were analyzed.

These findings indicate that DC maturation and cytokine production following TLR-activation are not altered in DC from CD patients homozygous for NOD2fs.

Figure 2



NOD2fs DC show no maturation or cytokine production after stimulation with MDP (A) Representative examples of the expression of CD80 and CD86 on DC from healthy controls (n=5), $NOD2^{+/+}$ CD patients (n=4) or NOD2fs CD patients (n=4) after stimulation with 5 µg/ml N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP) for a period of 24 hours. Medium control and MDP stimulation indicated by thin and thick lines, respectively. Filled histogram represents isotype-matched control mAb. Increase in mean fluorescence intensity of CD80 and CD86 after MDP stimulation was calculated. Differences between DCs from donors with intact NOD2 and NOD2fs DC were compared using Student's t-test (*, $P<0.05$). (B) IL-12, TNFα and IL-10 levels (pg/ml) in culture supernatant, comparing stimulation of cells with 100 ng/ml purified LPS or 5 µg/ml MDP for a period of 24 hours. No IL-12 could be detected (nd = not detectable) after stimulation with MDP, whereas some, but not all, control DC responded with the production of low levels of TNFα or IL-10.

MDP stimulation results in modest maturation and cytokine responses in control, but not NOD2fs DC

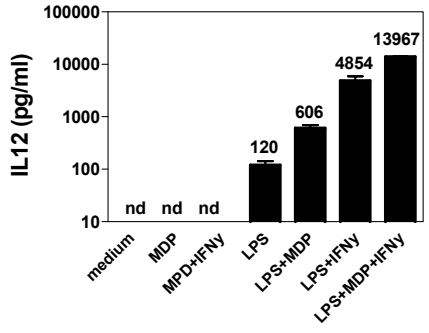
Next, the response of DC to the NOD2 ligand MDP was investigated. Based on initial titration experiments, a concentration of 5 µg/ml MDP was used. As shown in **figure 2A**, MDP treatment resulted in a weak but consistent upregulation of CD80 and CD86 on both DC from healthy donors and CD patients expressing wild-type NOD2. In contrast, NOD2fs DC invariably failed to increase the expression of these costimulatory molecules. Cytokine analysis further revealed that, in contrast to what is observed after TLR4 activation with LPS, no IL-12 was detected, and only very weak TNFα and IL-10 responses (mean 1.7% (0-11%) and 0.7% (0-3%) of LPS response, respectively) were observed following MDP stimulation in a number of control DC but not NOD2fs DC (**Fig. 2B**). These results indicate that NOD2 triggering alone does not induce significant cytokine production in DC, but does result in modest DC maturation that is completely abrogated in DC homozygous for the NOD2fs mutation.

Lack of cross-talk between NOD2 and TLR signals in NOD2fs DC

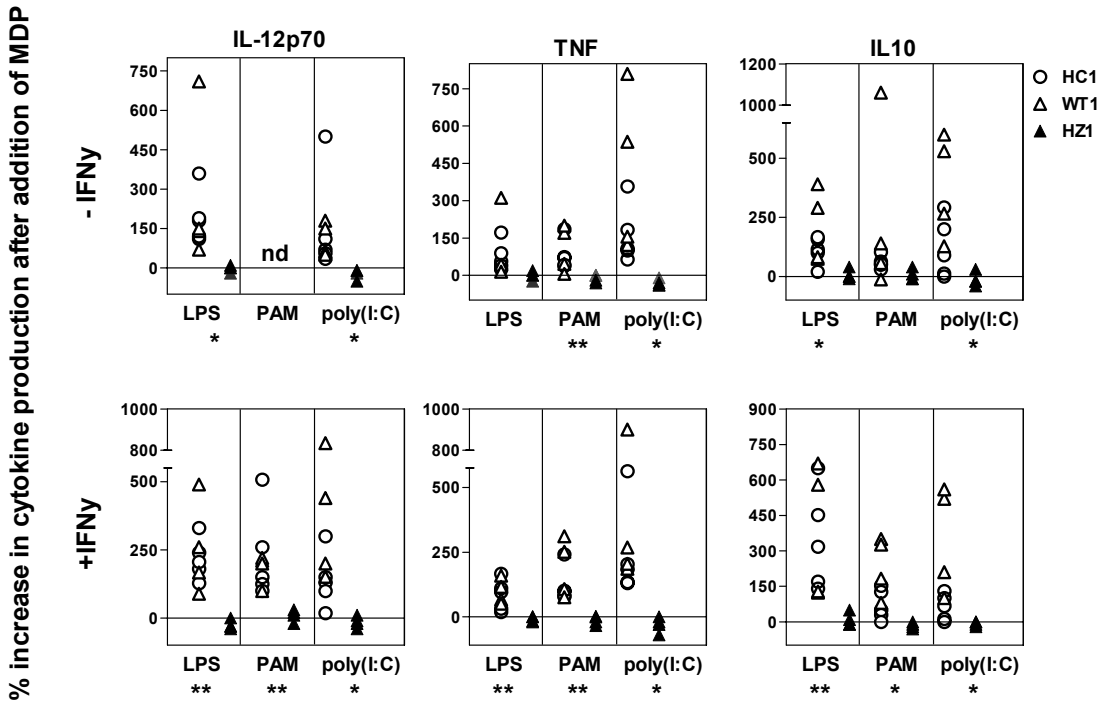
Others and we and we have recently reported that MDP can increase cytokine production when combined with TLR-activating stimuli [5, 17]. Considering the important role of IL-12 in CD, we first studied the release of this major Th1 driving cytokine in DC from healthy controls after activation with purified LPS. As shown in **figure 3A**, IL-12 was undetectable after MDP stimulation alone or in combination with IFNγ. In contrast, TLR4 activation using LPS resulted in an appreciable amount of IL-12 (120 pg/ml) that was further enhanced by IFNγ. In both the presence and absence of IFNγ, additional stimulation with MDP resulted in a significant increase in IL-12 as compared to LPS or LPS/IFNγ alone. Thus, the reported synergy between TLRs and NOD2 for the production of cytokines also applies for IL-12 release by human DC. Next, we studied the consequences of the NOD2fs mutation on cytokine production following combined NOD2/TLR stimulation in DC. Cells were treated with either LPS, PAM or poly(I:C) in the presence or absence of IFNγ with or without additional stimulation with MDP. IL-12, TNFα as well as IL-10 production was measured. Results are depicted as the % increase in cytokine levels, comparing TLR activation alone to TLR activation combined with MDP (**Fig. 3B**). In control DC, stimulation with TLR ligands in the presence of MDP resulted in a significant increase in IL-12, IL-10 and TNFα levels compared to TLR activation alone, although this effect was not significant for all cytokines measured or all TLR ligands used.

Figure 3

A



B



The NOD2fs mutation abrogates the synergy between TLR and NOD2 signals for release of cytokines
(A) IL-12 production by DC from healthy controls was measured after stimulation with 5 µg/ml MDP, 100 ng/ml purified LPS or a combination of both in the presence or absence of 400 U/ml IFNγ. Data shown are the mean + SD of triplicate measurements of one of four independent experiments. nd = not detectable. (B) IL-12, TNFα and IL-10 levels were measured following stimulation of DC from healthy controls (n=5), NOD2$^{+/+}$ CD patients (n=4) or NOD2fs CD patients (n=4) with 100 ng purified LPS, 10 µg/ml Pam3Cys or 20 µg/ml poly(I:C) alone or in combination with 5 µg/ml MDP for 24 hours. Experiments were performed without (upper panel) or with (lower panel) additional IFNγ stimulation. Displayed is the percentage increase in cytokine production as a result of the addition of MDP, compared to TLR stimulation alone. Differences in the observed increases in cytokine levels between control DC and NOD2fs DC were analyzed using Student's t-test (*, P<0.05 and **, P<0.01).

The synergistic effect of MDP stimulation on TLR induced cytokine release was completely absent in DC from NODfs patients. In the presence of IFNγ, there was a statistically significant difference for all cytokines measured after stimulation with the selected TLR-ligands and MDP, indicating that in a pro-inflammatory Th1 environment, the effect of NOD2fs could become even more apparent.

Together, these results demonstrate that the 3020insC mutation renders DC unable to mature in response to MDP and abrogates the synergy between NOD2 and TLRs for the production of both pro-and anti-inflammatory cytokines.

Discussion

Crohn's disease is associated with high levels of the NFκB-induced cytokines IL-12 and TNFα and monoclonal antibodies against these cytokines are effective therapies for CD [18-20]. Strikingly, decreased NFκB activation and defective cytokine responses have been reported in both human and mouse studies as a consequence of the CD-associated NOD2fs mutation providing evidence for a loss-of-function phenotype [1, 8, 21, 22]. However, two recent studies in mice challenged this view [6, 7]. Watanabe and colleagues suggested that NOD2 is a negative regulator of TLR2 induced Th1 responses, as measured by IL-12 production. Wild-type cells stimulated with the TLR2 ligand Pam3Cys plus MDP displayed lower IL-12 production than cells from $NOD2^{-/-}$ mice that were stimulated similarly [6]. In addition, Maeda *et al* developed a mouse model in which the NOD2fs variant was introduced into the mouse NOD2 locus and showed that mutant mice exhibited elevated NFκB activation and IL-1β secretion following MDP stimulation, suggesting that the NOD2fs mutation would lead to a gain-of-function phenotype [7]. This discrepancy between recent mouse and human data published necessitates further studies in, preferentially, human subjects. A lot of studies have used transfected human cell lines, but in these systems, NOD2 expression is well above physiological levels, with unknown outcome. Also, the use of (patient derived) peripheral blood mononuclear cells complicates identification of the effect of NOD2fs in specific cell-types within this mixed population.

DC are present in the intestinal mucosa where they sense the content of the intraluminal bacterial milieu, and they are crucial in the decision process between tolerance and immunity [12, 23]. We therefore decided to determine the consequence of the NOD2fs mutation in myeloid DC obtained from patients homozygous for this NOD2 polymorphism. Our results indicate that intact NOD2 is essential for the recognition of MDP by human DC and support the view that the NOD2fs mutation in

CD patients results in a loss-of-function phenotype in these cells. To avoid the unwanted effect of possible contamination with for instance endotoxins, we used only synthetic or highly purified TLR and NOD2 ligands during our study. DC from CD patients bearing the NOD2fs mutation responded with a normal upregulation of the costimulatory molecules CD80 and CD86 after TLR-activation but failed to do so in response to MDP. The production of IL-12p70, TNFα and IL-10 was not significantly different between DC from control and NOD2fs DC after TLR activation. MDP induced very low cytokine responses in some but not all DC carrying intact NOD2, while NOD2fs DC did not produce any cytokines following MDP stimulation alone. Finally, in control DC, stimulation with TLR ligands plus MDP enhanced the production of IL-12, TNFα and IL-10, compared to those levels obtained following TLR activation alone. This synergy between NOD2 and TLRs was completely absent in DC obtained from patients with the NOD2fs polymorphism. These results are in concordance with the recent findings by Van Heel *et al* [22] who showed that PBMCs from homozygous NOD2fs donors display significantly decreased TNFβ and IL-1β production following combined activation of TLRs and NOD2.

In the presence of IFNγ, that is known to increase NOD2 and TLR expression and function [24-26], the observed differences were even more significant. The absence of an increase in IL-12 production by simultaneous TLR2 and NOD2 activation in NOD2fs DC is at variance with findings reported in NOD2 deficient mice [6]. The reason for this discrepancy is currently unknown, but might be related either to differences between the NOD2fs mutation and complete absence of NOD2 or (species-related) differences between human DC and mouse splenocytes. In addition, other mutations in NOD2 might have different consequences, for instance in the case of Blau syndrome [27].

The inflammatory phenotype found in CD patients is hard to explain by a decreased TNFα and IL-12 production by NOD2fs DC after combined TLR/NOD2 activation. However, we now demonstrate that NODfs DC also produce decreased amounts of the anti-inflammatory cytokine IL-10 under these conditions. IL-10 is crucially involved in preventing excessive immune responses, including down-regulation of IL-12 and TNFα production [28]. We therefore postulate that deficient IL-10-mediated immune suppression is dominant over the reduction in TNFα and IL-12 levels that is also observed after TLR/NOD2 stimulation in NOD2fs DC. Since DC instruct the adaptive immune system via the release of specific cytokine combinations, a disturbed organization of this cytokine production could induce unwanted T-cell responses. Indeed, studies using therapeutic IL-10 administration or IL-10 deficient mice have demonstrated the importance of this cytokine in preventing mucosal inflammation [29,

30]. Furthermore, IL-10 is a multi-functional cytokine that is also crucial for the development of suppressor T-cells that are intricately involved in controlling intestinal immune responses [31-33]. NOD2fs related reduction in IL-10 levels could thus result in a defective counter-regulation of the effect of pro-inflammatory cytokines with concomitant Th1 responses and thereby contribute to the perpetuation of chronic inflammation characteristic for Crohn's disease.

Acknowledgments

The authors wish to thank M. den Brok, R. Sutmuller and R. Torensma for manuscript revision and T. Jansen, L. Jacobs and L. Toonen for technical assistance. M.G.N. was supported by a ViDi grant from the Netherlands Organization for Scientific Research.

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Chapter 5

Engagement of NOD2 has a dual effect on pro-IL-1β mRNA transcription and secretion of bioactive IL-1β

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European Journal of Immunology (2008) 38(1): 465-479

Summary

Synthesis and release of pro-inflammatory cytokines, such as IL-1β, play a crucial role in the intestinal inflammation that characterizes Crohn's disease (CD). Mutations in the NOD2 gene are associated with an increased risk of CD. Although it is known that NOD2 mediates cytokine responses to muramyl dipeptide (MDP), it is yet unclear whether NOD2 stimulation mediates only transcription of pro-IL-1β mRNA, or whether NOD2 is also involved in the activation of caspase-1 and release of active IL-1β. By investigating the response of MNC from CD patients homozygous for the 3020insC NOD2 mutation, we were able to show that NOD2 signaling after stimulation with MDP has a dual effect by activating proIL-1β mRNA transcription and inducing release of bioactive IL-1β. Because NOD2 engagement amplifies TLR stimulation, we investigated whether activation of caspase-1 by MDP is involved in the NOD2/TLR synergism. The synergy in IL-1β production between NOD2 and TLRs is mediated at post-translational level in a caspase-1-dependent manner, which indirectly suggests that NOD2 also induces caspase-1 activation. In contrast, the synergy in TNFα production after stimulation with MDP and LPS is induced at transcriptional level. This demonstrates that both caspase-1-dependent and -independent mechanisms are involved in the synergy between NOD2 and TLRs.

Introduction

NOD-like receptors (NLRs) are intracellular receptors for bacterial peptidoglycan, which complement the recognition of pathogen-associated molecular patterns (PAMPs) by membrane-bound Toll-like receptors (TLRs) [1, 2]. NOD2 is a member of the NACHT-LRR (NLR) receptor family, which recognizes muramyl dipeptide (MDP), the minimal motif of peptidoglycan of both Gram-positive and Gram-negative bacteria [3]. Mutations in the NOD2 gene are associated with Crohn's disease [4, 5], but how NOD2 exactly acts in the pathogenesis of this auto-inflammatory disease is unclear [6, 8]. Therefore, a better understanding of the intracellular events induced by the interaction between NOD2 and peptidoglycan is crucial for both the insight into recognition of Gram-positive pathogens by the innate immune system, and for the pathogenesis of the inflammatory reactions in Crohn's disease.

Activation of human mononuclear cells (MNC) by MDP leads to production of pro-inflammatory cytokines, especially interleukin-1β (IL-1β) [7, 9]. IL-1β is produced as pro-IL-1β a 31–34-kDa inactive form of the cytokine, which is later cleaved by caspase-1 to the bio-active 17-kDa IL-1β [10]. This is followed by IL-1β excretion in microvesicles into the extracellular environment [11]. Apparently, MDP is capable of inducing all three steps, but it is unclear whether NOD2 alone or other receptors are involved in one or more of these steps of IL-1β production. It has been proposed that several of the NLR family members are able to recognize MDP, most notably NOD2, NALP3, and NALP1, and that they execute different functions necessary for cytokine production. In this concept, recognition of MDP by NOD2 would mainly activate NF-kB and thereby gene transcription of pro-IL-1β, whereas NALP3/NALP1-mediated recognition of MDP leads to caspase-1 activation and the subsequent release of the active IL-1β form [12-14]. However, there are controversies regarding this mechanism of IL-1β production by MDP. The caspase-recruitment domain (CARD) of NOD2 interacts with the serine**/**tyrosine kinase RIP2, leading to NF-kB translocation and transcription of mRNA for pro-inflammatory cytokines [15-17]. However, it is also known that RIP2 interacts with the CARD domain of caspase-1 [18, 19]. Thus, one could envisage that NOD2 engagement by MDP could activate caspase-1 and lead to the release of mature IL-1β, without the need for a secondary interaction with NALP3**/**NALP1.

In the present study, we aimed to assess whether recognition of MDP by NOD2 is important for both the induction of pro-inflammatory cytokines gene transcription, as well as for the activation of caspase-1 and IL-1β release, by comparing the response of MNC from patients with Crohn's disease homozygous for the 3020insC NOD2 mutation

(NOD2fs) with the response of MNC isolated from individuals with wild-type NOD2 allele (NOD2wt). In addition, because MDP is able to amplify the TLR stimulation through a NOD2-dependent pathway [9, 20], we also investigated whether activation of caspase-1 by MDP is involved in the NOD2/TLR synergism.

Materials and Methods

Reagents

Synthetic Pam3Cys was purchased from EMC Microcollections (Tubingen, Germany). LPS (*E. coli* serotype 055:B5 was purchased from Sigma (St. Louis, MO) and an extra purification step was performed as previously described [41]. The purified LPS was tested in TLR4$^{-/-}$ mice for the presence of contaminants and it did not have any TLR4-independent activity. Synthetic MDP was obtained from Sigma. The reversible caspase-1 inhibitor (ICE-i) Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone (YVAD) was purchased from Alexis Biochemicals (San Diego, CA) and solubilized in dimethyl sulfoxide (DMSO) at 10 mg/ml. The ICE-i was diluted to the desired concentration in RPMI.

Genotyping of *NOD2* variants

Blood was collected from 154 patients with Crohn's disease and 10 healthy volunteers. PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50µl reaction volumes containing 100-200 ng genomic DNA as previously described [2]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems). Seven patients with Crohn's disease were found homozygous for the 3020insC mutation, and four of them were further investigated in the cytokine studies. As control groups, four patients with Crohn's disease and four healthy volunteers homozygous for the wild-type *NOD2* allele were included.

Isolation of mononuclear cells and stimulation of cytokine production

After obtaining informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10 ml EDTA tubes (Monoject). Isolation of mononuclear cells (MNC) was performed as described elsewhere [3], with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and suspended in culture medium (RPMI 1640) supplemented with gentamicin 10 µg/ml, L-glutamine 10 mM and pyruvate 10 mM. The cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to $5x10^6$ cells/ml. $5x10^5$ MNC in a 100 µl volume were added to round-bottom 96-wells plates (Greiner) and incubated with either 100 µl of culture medium (negative control), or the various stimuli: LPS (1 ng/ml), MDP (10 nM), or combinations of MDP and LPS at various concentrations. In separate experiments, inhibitors (ICE-i (20 µM) or IL-1Ra (10 µg/ml)) were added 10 minutes before stimulation. The stimuli were checked for the contamination with LPS in the LAL assay and found to be negative. After 24h, the supernatants were collected and stored at -70°C until assayed. To investigate the role of NOD2 for the release of IL-1β, PBMC were initially stimulated for 4h with LPS (1 µg/ml). After 4h, supernatants were collected and medium containing 1 mM ATP was added to the cells for another 15 min. The LPS-dependent IL-1β production during the first 4h and the ATP-dependent IL-1β secretion after the additional 15 minutes was assessed in the supernatant.

Cytokine measurements

Human TNFα concentrations were determined by specific ELISA [42]. IL-1β, proIL-1β and IL-10 were measured by commercial ELISA kits (R&D Systems, and Pelikine Compact, Sanquin), according to the instructions of the manufacturer.

Quantitative PCR

MNC were stimulated as described above, after 4 hours the supernatant was removed and the cells resuspended in 200 µl RNAzolB RNA isolation solvent (Campro Scientific) and frozen at -80˚C for storage. mRNA was isolated following the manufacturer's protocol. The amount and quality of mRNA were determent by spectrophotometry and analyzed by agarose gel electophoresis for DNA contamination. cDNA was synthesized from 1000 ng of total RNA using SuperScript^{tm} Reverse Transcriptase (Invitrogen Corp.; 18064-014). Relative mRNA levels were determined using the Biorad I-Cycler and SYBR Green method (Invitrogen Corp.; S7563) [43]. The following primers were used:

IL-1β forward (5′-TGGCCCAGGCAGTCAGA-3′); IL-1β reverse (5′-GGTTTGCTACAACATGGGCTACA-3′) TNFα forward (5′-GCCCTAAACAGATGAAGTGCTC-3′); TNFα reverse (5′-GAACCAGCATCTTCCTCAG-3′) B2M forward (5′-ATGAGTATGCCTGCCGTGTG-3′); B2M reverse (5′-CCAAATGCGGCATCTTCAAAC-3′)

Primers were obtained from Biolegio, Malden, The Netherlands. Mean relative mRNA expression from at least 2 replicate measurements was calculated using Biorad iCycler IQ software. Values are expressed as fold increase to mRNA levels of unstimulated cells.

Western blots for pro-IL-1β and IL-1β

Human MNC were stimulated as described above. After 4 hours stimulation, supernatant was removed and cells were lysed in 25 µl of ice-cold lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.1% SDS and protease inhibitors (Sigma*Fast*, Sigma). Lysates of 4.10^6 cells were pooled. Samples were 'taken up' in 25 µl sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% β-mercaptoethanol, 10% glycerol and 0.5 mg/ml of bromophenol blue), were separated by SDS-PAGE and were blotted onto polyvinyldifluoride membranes (Millipore). Membranes were blocked in 0.1% Tween-20 in Tris-buffered saline containing 5% nonfat dry milk and were incubated overnight with antibody against cleaved IL-1β (Cell Signaling) in 1% BSA and 0.1% Tween-20 in Tris-buffered saline. Horseradish peroxidase−conjungated secondary antibodies were visualized with Lumilite plus (Boehringer-Mannheim).

Statistical analysis

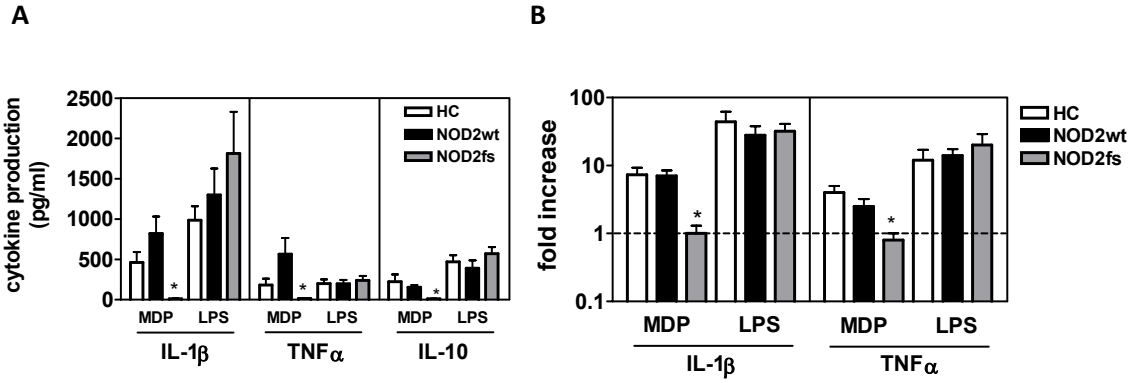
All experiments were performed at least in duplicate with blood obtained from 4 Crohn's disease patients bearing the NOD2fs mutation, 4 Crohn's disease patients with the wild-type NOD2 genotype, as well as 4 healthy volunteers. Synergy was expressed as ratio of cytokine response of ligand in combination with MDP divided by the sum of cytokine responses obtained with each ligand alone. The differences in cytokine production between groups were analyzed by Mann-Whitney U test or Wilcoxon, and where appropriate by Kruskal-Wallis ANOVA test. For all other comparisons the Student's t test was used. The level of significance between groups was set at $p<0.05$. The data are given as means $\pm$ SEM.

Results

MDP induces NOD2-dependent transcription of cytokine genes

In MNC of Crohn's disease patients with a homozygous 3020insC mutation (NOD2fs), cytokine production after stimulation with MDP was completely abolished, whereas the cytokine production after stimulation with the TLR4 ligand LPS was normal compared to healthy volunteers (HC) and Crohn's disease patients bearing the wild type allele (NOD2wt) (**Fig. 1A**). This impaired cytokine response to MDP is caused by a transcriptional defect, since MDP was found to increase mRNA in MNC of healthy volunteers and Crohn patients without NOD2 mutations of IL-1β and TNFα, but not in patients homozygous for the 3020insC mutation (**Fig. 1B**).

Figure 1



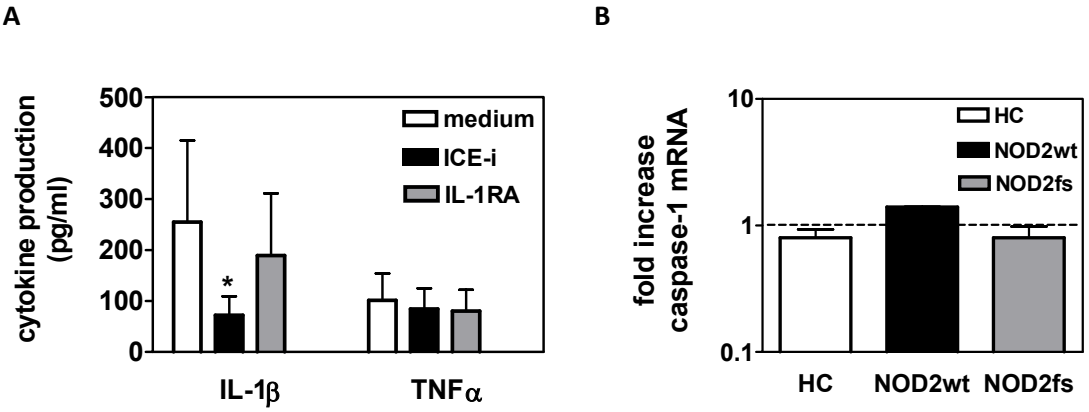
MDP induces NOD2 dependent cytokine production and transcription of pro-IL-1β and TNFα in MNCs. MNCs of 5 healthy controls (HC), 5 Crohn's disease patients without NOD2 mutations (NOD2wt) and 4 patients homozygous for the 3020insC mutation (NOD2fs) were stimulated with LPS (10 ng/ml) or MDP (100 nM). Cytokines were measured by ELISA in the supernatant after 24 hours incubation at 37°C (panel A). Quantitive measurement of mRNA levels of IL-1β and TNFα was performed by real-time PCR and expressed as fold increase compared to unstimulated cells. Cells were lysed in RNAzolB after 4 hours incubation at 37°C (panel B). Data presented as means ± SEM and compared by Mann-Whitney U test (*p< 0.05).

Induction of IL-1β by MDP is caspase-1 dependent

Various bacterial stimuli can induce mature IL-1β production, which requires both transcription of pro- IL-1β mRNA and post-translational processing by caspase-1. We found that IL-1β production in MNC after stimulation with MDP was blocked after inhibition of caspase-1 with YVAD (ICE-i) (**Fig. 2A**). This indicates that the post-

translational processing of pro-IL1β by caspase-1 is important for the IL-1β production by MDP. Inhibition of the IL-1R with IL-1Ra did not block the IL-1β production (Fig 2A). Thus, an autocrine feedback of IL-1β inducing IL-1β, as shown previously using LPS as a stimulus [21], does not seem to play a major role in MDP-induced IL-1β. During the exposure to MDP, caspase-1 mRNA levels did not increase compared to unstimulated cells, as shown in **figure 2B**. Since caspase-1 activity is not regulated at transcriptional level, the regulation of caspase-1 activation is likely post-translational by enzymatic cleavage of pro-caspase-1 [22].

Figure 2



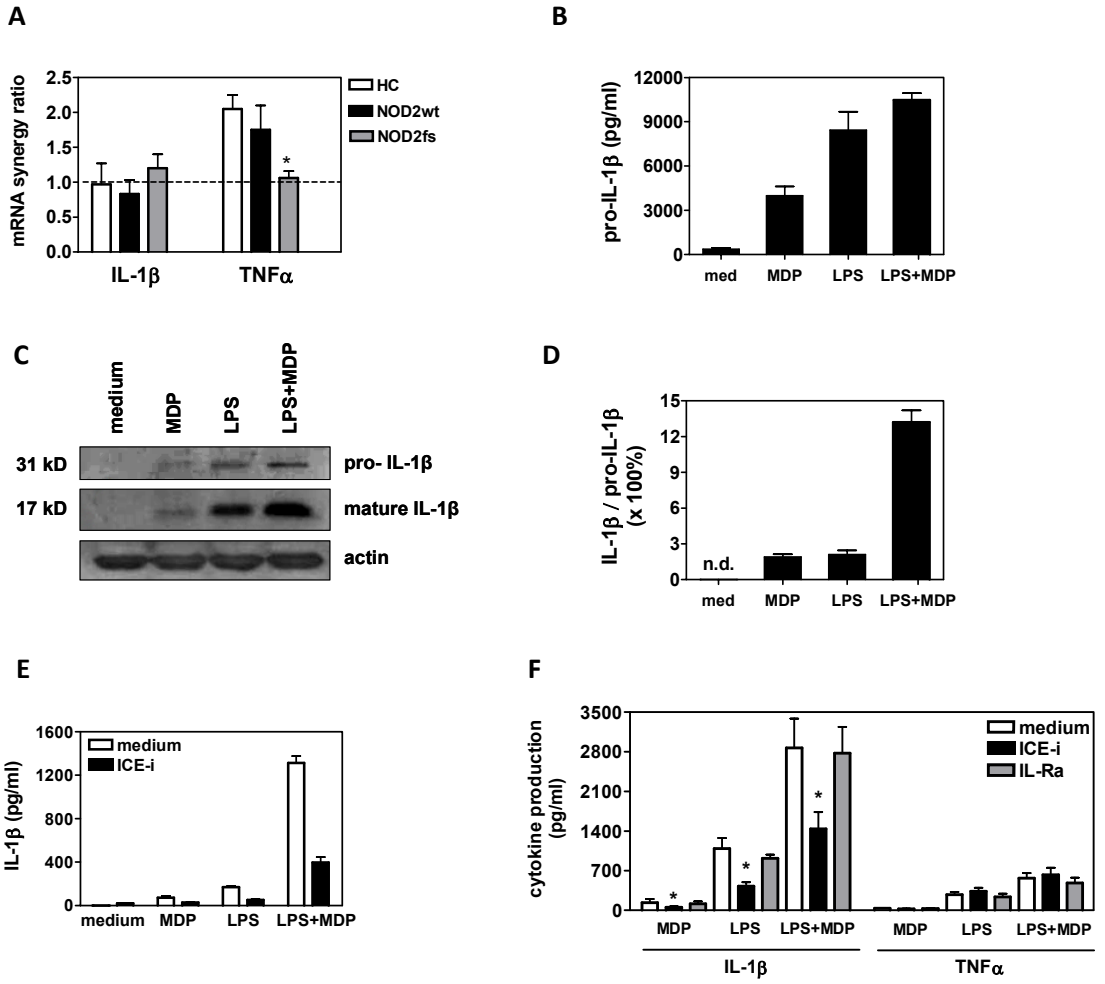
MDP activates caspase-1 but does not enhance transcription of caspase-1 mRNA. Stimulation of MNC of 5 healthy volunteers with MDP (100 nM) during inhibition of caspase-1 with ICE-i (20 μM) or the IL-1-receptor with IL-1Ra (10 μg/ml). Cytokines were measured by ELISA in the supernatant after 24 hours incubation at 37°C (panel A). MNCs of 4 healthy controls (HC), 4 Crohn's disease patients without NOD2 mutations (NOD2wt) and 4 patients homozygous for the 3020insC mutation (NOD2fs) were stimulated MDP (100 nM). After 4 hours incubation at 37°C, cells were lysed in RNAzolB. Quantitative measurement of mRNA levels of caspase-1 was performed by real-time PCR and expressed as fold increase compared to unstimulated cells (Panel B).

The synergism between NOD2 and TLRs for IL-1β is caspase-1 dependent

Stimulation of cells with MDP and the TLR4 ligand LPS induces cytokines in a synergistic fashion [9, 20]. Because MDP can activate caspase-1 and induce mature IL-1β, we investigated whether caspase-1 is important for this synergy. Induction of IL-1β mRNA in MNCs of HC, NOD2wt and NOD2fs was not increased after stimulation with MDP and LPS compared to LPS alone (**Fig. 3A**). In addition, there was no synergy in the production of pro-IL-1β production, as assessed both by a specific pro-IL-1β ELISA (**Fig.**

3B) or Western blots (**Fig. 3C**). However, mature IL-1β was secreted in a synergistic way (**Fig. 3E**). The increased ratio between intracellular pro-IL-1β and secreted IL-1β indicates that the synergistic production of IL-1β after stimulation with MDP and LPS is established either by extra cleaving of pro-IL-1β or/and by increased release of bioactive IL-1β (**Fig. 3D**).

Figure 3



Synergism between TLRs and NOD2 for the release of IL-1β is exerted at post-transcriptional level. MNCs from four healthy volunteers were stimulated with MDP (100 nM), LPS (10 ng/ml) or a combination of MDP and LPS. After 4 hours incubation at 37°C, cells were lysed in RNAzolB. Quantitive measurement of mRNA levels of IL-1β and TNFα was performed by real-time PCR and expressed as fold increase compared to LPS treated cells (Panel A). Pro-IL-1β production after 4h stimulation was assessed by a specific ELISA (Panel B) or Western blots (Panel C). Similarly, IL-1β concentrations were assessed by ELISA (Panel E). The percentages of mature IL-1 secreted from cells stimulated with MDP, LPS or the combination LPS+MDP are presented in Panel D. Stimulation of MNCs of 9 healthy volunteers with LPS (10 ng/ml) in combination with MDP (100 nM) during inhibition of caspase-1 with ICE-i (20 μM) or the IL-1R with IL-1Ra (10 μg/ml). Cytokines were measured by ELISA in the supernatant after 24 hours incubation at 37°C (Panel E). Data presented as means ± SEM and compared by Mann-Whitney U test (*$p < 0.05$).

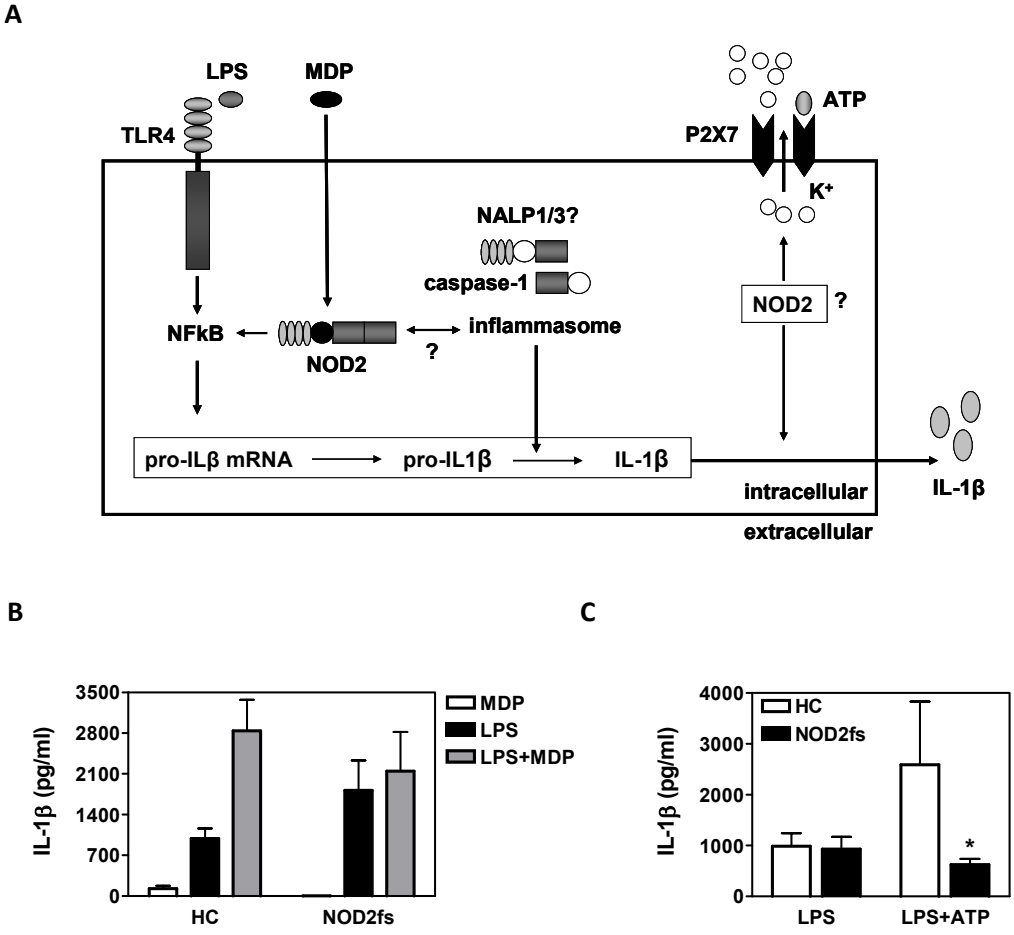
In support of the notion that processing of pro-IL-1β is the level at which the synergism between NOD2 and TLRs takes place, when caspase-1 was inhibited with YVAD (ICE-i) the synergy declined, showing that caspase-1 is responsible for the synergy of IL-1β production after stimulation with MDP and LPS (**Fig. 3F**). In contrast to IL-1β, the induction of TNFα mRNA in MNCs of HC and NOD2wt increased after stimulation with MDP and LPS compared to LPS alone in the same ratio as TNFα in the supernatant. In MNCs of NOD2fs no increase of TNFα mRNA and TNFα was found (**Fig. 3A**). Inhibition of caspase-1 did not influence the TNFα production or synergy (**Fig. 3F**). Blocking the IL-1R with IL-1Ra had no effect on the synergistic production of IL-1β and TNFα, indicating that autocrine stimulation of IL-1R cannot explain the synergy (**Fig. 3F**). These data show that the synergy in TNFα production after stimulation with MDP and LPS is induced at transcriptional level, whereas the synergy in IL1β production is regulated at post-translational level by activation of caspase-1.

Activation of caspase-1 and release of bioactive IL-1β by MDP is NOD2 dependent

To test whether MDP uses another receptor than NOD2 for the activation of caspase-1 (*e.g.* NALP3), and in the absence of a reliable caspase-1 p10 Western-blot methodology in human primary cells, we designed an indirect functional assay to evaluate this hypothesis. If MDP would activate capsase-1 through NALP3 or NALP1, one would expect that although MDP is unable by itself to induce IL-1β in cells from NOD2fs patients, due to its inability to activate transcription, it would still be able to amplify IL-1β production induced by a TLR ligand (**Fig. 4A**). This would happen because of the intracellular proIL-1β induction by the TLR agonist, while processing of IL-1β at the level of caspase-1 activation would be amplified by the MDP-NALP3/NALP1 interaction (**Fig. 4A**). However, the data presented in **figure 4B** strongly argue against this hypothesis. These data show clearly that MDP was unable to amplify IL-1β production when cells of Crohn's disease patients with NOD2fs were stimulated with LPS, suggesting that NOD2 is necessary for the caspase-1 activation by MDP.

In order to investigate the role of NOD2 for the release of active IL-1β from the cells, we tested the effects of the NOD2-deficiency in an ATP/LPS stimulation assay. When ATP was added to LPS-primed cells, a significant increase in the IL-1β secreted from the cells bearing only the wild-type NOD2 allele was observed. In contrast, the ATP-dependent IL-1β release was severely impaired in the cells isolated from individuals homozygous for the NOD2fs mutation (**Fig. 4C**). IL-1β production after LPS stimulation alone was not different between NOD2wt and NOD2fs individuals (**Fig. 4C**).

Figure 4



Activation of caspase-1 by MDP is NOD2 dependent. Putative scheme of activation of the inflammasome by MDP, in which the MDP/NOD2 interaction activates transcription, processing and release of IL-1β. A potential interaction of NOD2 with NALP3/NALP1 for the activation of the inflammasome is still unclear (Panel A). Stimulation of MNCs of 4 healthy controls (HC) and 4 patients homozygous for the 3020insC mutation (NOD2fs) with MDP (100 nM), LPS (10 ng/ml) or a combination of MDP and LPS. Cells were incubated for 24 hours at 37°C and IL-1β was measured in the supernatant by ELISA (Panel B). Stimulation of MNCs from healthy controls or patients homozygous for the 3020insC mutation (NOD2fs) for 4h using LPS (1 µg/ml) with or without an additional incubation with ATP (1 mM) for 15 min (Panel C). Data presented as means ± SEM and compared by Mann-Whitney U test (* $p < 0.05$).

Discussion

In this study, we show that NOD2 has dual effects on both pro-IL-1β mRNA transcription and the release of bioactive IL-1β from the cells. In addition, indirect

functional tests on the role of caspase-1 for the synergistic effects between NOD2 and TLRs also suggest a role for NOD2 for the activation of caspase-1 by MDP, the active component of bacterial peptidoglycan.

The regulation and activation of caspase-1 and thereby the regulation of the production of mature IL-1β, followed by its secretion from the cells, is a rapid evolving field of research. The importance of understanding these crucial steps in IL-1β processing and release is stressed by the role of IL-1β in virtual all forms of inflammation and especially in auto-inflammatory diseases, such as familial Mediterranean fever (FMF), hyperimmunoglobulinemia D syndrome (HIDS) and Muckle-Wells syndrome [22]. Targeting IL-1β in these disorders has shown spectacular results [23-27], and targeting caspase-1 could lead to new treatment strategies. Some reports indicate that caspase-1 activity is partially regulated at transcriptional level [28]. However, our results show that stimulation with MDP did not result in an increase expression of caspase-1 mRNA. This is in line with the current opinion that caspase-1 activity is mainly regulated at posttranslational level [29, 30]. In this view, caspase-1 is activated by close proximity mechanism in a multimeric protein platform, called the inflammasome. It is unclear which pathway directly activates the inflammasome and how many different of these platforms exists. The most studied inflammasomes consist of NALP3/ASC/cardinal/caspase-1 and NALP1/ASC/caspase-1/caspase-5. NALPs belong to the same protein family as NODs and CIITA, which is named the NLR (NACHT-LRR receptors or NOD-like receptors) family. All NLRs have a NACHT domain which is involved in forming multimers. In addition, some members have a CARD used for interaction with other CARD containing proteins, such as caspases. Finally, the LRR domain can interact with PAMPs (e.g. MDP interaction with LRR of NOD2). An interesting hypothesis is that the interaction of a PAMP with the LRR of a NLR in the inflammasome can activate caspase-1. Indeed, Martinon et al proposed that MDP interacts with the LRR of NALP3 in cell-lines and can activate the inflammasome in human macrophages [12]. Similarly, a recent study has suggested that NALP-1 is also an MDP receptor activating caspase-1 [14].

In human MNC we confirm the activation of the inflammasome by MDP, indicated by the release of mature IL-1β after stimulation with MDP. Importantly, indirect proof based on a NOD2/TLR stimulation assay suggests that activation of the caspase-1 seems to occur in a NOD2-dependent manner. If MDP would activate caspase-1 independently of NOD2, stimulation of cells lacking a functional NOD2, such as NOD2fs MNC, with LPS and MDP should result in an amplification of LPS-induced IL-1β production through MDP-NALP3/NALP1 dependent mechanisms. In NOD2-deficient

cells LPS induces pro-IL-1β and activation of caspase-1, and MDP should amplify caspase-1 activation and IL-1β release in a NOD2-independent, NALP3- or NALP1-dependent manner. However, the data presented here clearly show that this is not the case: no increase in IL-1β production was documented when NOD2fs cells are stimulated with LPS and MDP at the same time. This suggests that NOD2 has a non-redundant function for caspase-1 activation by MDP (Fig 4A). However, it cannot be excluded that NOD2 also requires the presence of NALP3 and/or NALP1 and collaborate with one or both of these molecules for the activation of caspase-1. A recent study by Pan and colleagues showing that both NOD2 and NALP3 are necessary for IL-1β secretion by MDP in murine macrophages gives weight to this hypothesis [31]. Unfortunately, the lack of a reliable Western-blot assay for the activated caspase-1 p10 in human primary monocytes has precluded us to obtain direct evidence of the role of NOD2 for the caspase-1 activation. However, by using a well-established model of IL-1β release after LPS priming of cells, followed by stimulation with the K^+-channel activator ATP, we were able to demonstrate an important role for NOD2 in the release of active IL-1β. This is in line with a recent study showing that NALP3, another member of the NLR family, binds ATP [32].

Some of the differences between our findings and the study of Martinon et al [12] in terms of IL-1β induction by purified LPS could be explained by the use of different cell types: freshly MNC produce mature IL-1β after stimulation with LPS, whereas monocytes-derived macrophages do not. Furthermore, it has to be realised that data from cell-lines and over expression models are not always compatible with data obtained from freshly isolated human cells. In addition, differences exist between NALP3-/- mice and human cells [12, 33, 34], and also between NOD2-/- mice and cells obtained from Crohn's disease patients homozygous for the NOD2fs allele [7, 35].

The unique role of NOD2 as key receptor of MDP for the induction of NF-κB dependent transcription of mRNA of cytokines is underlined by showing that mRNA of IL-1β and TNFα in NOD2fs cells is not increased after stimulation with MDP. Subsequently, the production of the cytokines is abolished. Our quantitative data by real-time PCR, obtained in primary cells, are supported by the results obtained by other groups [36-38].

When MNC are stimulated with low dose MDP and the TLR4 ligand LPS, a remarkable synergy in cytokine production is apparent in MNC from individuals with a intact NOD2 [9, 20]. A potential role for caspase-1 in the synergy between TLR4 and NOD2 could be envisaged, since MDP can activate caspase-1 and induce IL-1β, which is known to

induce IL-1β production in an autocrine fashion through stimulation of the IL-1R [21]. In addition, IL-1β can induce the production of other cytokines, like IL-10 and IL-6 [39]. Indeed, the synergistic production of IL-1β by MDP and LPS is caspase-1dependent, and not regulated at transcriptional level. However, in line with other studies, we show that the mechanism of synergy for TNFα is exerted at a transcriptional level [40].

In conclusion, we demonstrate that NOD2 is non-redundant for both the production of IL-1β mRNA and the IL-1β release from activated cells. In addition, indirect data supports a role for NOD2 in the activation of caspase-1 in human MNC after stimulation with MDP. Furthermore, caspase-1-dependent mechanisms are responsible for the synergistic effect on IL-1β production between NOD2 and TLRs. Currently it is unclear whether NOD2 is part of an inflammasome protein complex or if it directly activates caspase-1. Further investigation is needed to unravel the exact role role of NOD2, but the picture emerges that NOD2 is the key receptor for MDP that executes its function at different levels in the pathways of cytokine production.

Acknowledgments

M.G.N. was supported by a VIDI grant from The Netherlands Organization for Scientific research.

There is no conflict of interest.

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Chapter 6

Echovirus infection causes rapid loss-of-function and cell death in human dendritic cells

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Cellular Microbiology (2007) 9(6): 1507-1518

Abstract

Coxsackie B viruses (CVB) and Echoviruses (EV) form a single species; Human enterovirus B (HeV-B), within the genus *Enterovirus*. Although HeV-B infections are usually mild or asymptomatic, they can cause serious acute illnesses. In addition, HeV-B infections have been associated with chronic immune disorders, such as type 1 diabetes mellitus and chronic myocarditis/dilated cardiomyopathy. It has therefore been suggested that these viruses may trigger an autoimmune process. Here, we demonstrate that human dendritic cells (DCs), which play an essential role in orchestration of the immune response, are productively infected by EV, but not CVB strains, *in vitro*. Infection does not result in DC activation or the induction of anti-viral immune responses. Instead, EV infection rapidly impedes Toll-like receptor mediated production of cytokines and upregulation of maturation markers, and ultimately causes loss of DC viability. These results describe for the first time the effect of EV on the function and viability of human DCs and suggest that infection of DCs *in vivo* can impede regulation of immune responses.

Introduction

Dendritic cells (DCs) are the most efficient antigen-presenting cells. They are present in virtually all organs and tissues throughout the body and are potent initiators of immunity via activation of (naïve) T- and B-cells, but also regulate tolerance to self-antigens in order to prevent auto-immunity [1]. Via expression of a broad array of pattern recognition receptors, for instance Toll-like Receptors (TLRs) and C-type lectins, DCs can sense the presence of a large variety of pathogens, such as bacteria and fungi [2, 3]. Triggering of TLRs results in DC maturation and cytokine production, enabling them to regulate T cell activation and differentiation [4].

Also in the event of viral infection, DCs play a crucial role in the induction of anti-viral immune responses. Besides responding to virus-associated structures, such as ssRNA and dsRNA [5, 6], DCs can take up virus particles or (apoptotic) virus-infected cells. This enables presentation of viral peptides in the context of MHC-II or MHC-I [7, 8] and the induction of potent anti-viral T cell responses. However, viruses have co-evolved with their host to evade the immune system by several mechanisms. Considering the central role of DCs in the immune system, it is not surprising that these cells are often targeted by viruses. For instance, infection of DCs with cytomegalovirus results in defective maturation, hampering the activation of naïve T cells [9, 10]. In addition, measles virus impedes DC derived IL-12 production, while increasing the production of the anti-inflammatory cytokine IL-10 [11]. This mechanism could possibly explain the observed immune suppression as a result of measles virus infection. Thus, although DCs are important contributors to anti-viral immunity, virus infection can impair DC function and immune responses, potentially leading to persistent infections.

Coxsackie B viruses (CVB) and Echoviruses (EV) are small (20-30 nm), non-enveloped enteroviruses that are closely related and classified as a single species, Human enterovirus B (HeV-B), within the *Enterovirus* genus of the *Picornaviridae* family. They mainly differ in their capsid-coding region and thus in receptor usage. They are acid stable and primarily cause infection of the gastrointestinal tract. Infection usually remains limited to the intestine and causes mild disease or remains asymptomatic. Incidentally, however, infection spreads via the blood stream to affect specific target organs as the brains, pancreas and heart where it can give rise to severe and potentially fatal illnesses such as (meningo)encephalitis, pancreatitis and myocarditis [12]. In addition, it has been suggested that CVB and EV play a role in the pathogenesis of chronic immune disorders like type 1 diabetes mellitus (T1D) [13-15], primary

Sjögren's syndrome [16] and chronic myocarditis/dilated cardiomyopathy [17-19]. Hence, it has been proposed that HeV-B infections may trigger autoimmunity [20-22].

To date, no information is available concerning the direct effect of HeV-B infections on DCs. We investigated the capacity of several CVB and EV strains to infect human DCs and analyzed the potential effects on DC function. We show here that the ability of these viruses to infect and replicate in human monocyte-derived DCs is restricted to EV strains only. EV infection does not lead to DC activation, but instead results in a rapid loss of responsiveness to TLR ligands and induction of cell death.

Materials & Methods

Virus stocks and purification

Reference strains Echovirus 1 Farouk (EV1 Farouk), EV7 Wallace, EV8 Bryson, EV9 Hill and Coxsackievirus B4 strain Tilo (CVB4 Tilo) were obtained from the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). CVB3 Nancy and CVB4 Edwards2 were kindly provided by R. Kandolf (University of Tübingen, Germany) and J.W. Yoon (University of Calgary, Canada), respectively. Production of virus stocks and virus titrations were performed on buffalo green monkey cells. Cells were grown in minimal essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a 5% CO_2-incubator. After infection, cells were incubated until complete cytopathic effect was observed. Virus was released by 3 successive cycles of freezing and thawing and cell debris was removed by centrifugation for 5 min at 3000 rpm. For purification, viral particles were pelleted by centrifugation through a 30% sucrose cushion in a Beckman SW28 rotor for 6 h at 25,000 rpm. Virus was resuspended in PBS and virus titers were determined by endpoint titration. Serial 10-fold dilutions were tested in 96-well microtiter plates and fifty percent Tissue Culture Infective Doses ($TCID_{50}$) were calculated as described before [59].

Plasmids

The CVB3 infectious cDNA clone used in this study, p53CB3/T7, has been described previously [59]. The EV9 Hill infectious cDNA clone [63] was generously provided by B. Nelsen-Salz (Virology Institute, University of Cologne, Germany).

Isolation of mononuclear cells and generation of monocyte-derived DC

Buffy coats (Sanquin bloodbank Nijmegen) were diluted using PBS containing 0.45% sodium citrate (dilution solution). Mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (1.077 g/ml; Axis Shield PoC AS) according to manufacturer's instructions. Cells were washed once in dilution solution to remove Lymphoprep remains and further washed using ice cold dilution solution containing 0.1% BSA until clear supernatant was obtained. After counting, cells were resuspended in RPMI 1640 (Invitrogen Life Technologies) containing 2% human serum at a density of 12.5 to15 X 10^6 cells/ml and plated out in T75 culture flasks (Costar) at 37°C for a period of 1 h. Non-adherent cells were removed by washing thoroughly with PBS and the adherent cell fraction was cultured in RPMI 1640 supplemented with 100 U/ml antibiotic-antimycotic (Invitrogen), 10 mM L-glutamine and 10% FCS. To generate monocyte-derived DC, 300 U/ml IL-4 and 400 U/ml GM-CSF (Strahtman) was added to the culture medium. Medium was refreshed and complete cytokines added at day 3. On day 6, immature DCs were harvested using cold PBS and directly used as described below. To obtain mature DCs, cells were stimulated with LPS (100 ng/ml) or R848 (4 µg/ml) for a period of 24 h.

Infection of monocytes and DCs

Monocytes were obtained by incubation of PBMC in RPMI with 2% HS in 96-wells plates for 1 h at 37 °C. Nonadherent cells were removed by washing thoroughly with PBS and the adherent cells were infected with virus at the indicated MOI in SF medium. After incubation for 60 min at 37°C, cells were washed 3

times with PBS and 150 µl full medium was added to the wells. For replication analysis, viruses were released at specific times post infection by 3 successive freeze-thaw cycles. Immature and mature DCs were harvested using cold PBS, washed and infected at an MOI of 10 in SF RPMI, unless indicated otherwise. After a 60 min incubation at 37^{o}C, cells were washed 3 times in an excess volume of PBS and plated out in 96-wells plates in full medium. Titrations were done as described above.

Western blot analysis

At 4 and 8 h post infection, cells were lysed and lysates stored at -20°C. Equal amounts of protein were separated by 12.5% SDS-PAGE, electroblotted onto nitro-cellulose membranes (Bio-Rad), followed by probing with rabbit polyclonal antiserum raised against the first 60 aa of CVB3 3A (anti-3A[1-60]) [64] in a 1:300 dilution. Because the 3A proteins of CVB3 and EV9 are nearly identical (98% identity, 100% similarity), this antibody recognizes the 3A protein of both viruses. After 3 washes, membranes were incubated with a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulins (Dako Diagnostika). Analysis was performed using the Lumi-Lightplus Western blotting substrate (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Immune fluorescence

Immature DC (day 6) were harvested using cold PBS and infected with CVB3 or EV9 at an MOI of 10 in SF medium. After a 7 h infection period cells were washed 2 times in PBS, resuspended in SF medium and 50 X 10^{3} cells were allowed to adhere to poly-L-lysine coated coverslips for 1 h at 37^{o}C. Cells were washed, fixed with 1% paraformaldehyde (PFA) at room temperature (RT) for 20 min and blocked in PBS with 3% BSA, 10 mM glycine and 2% human serum (blocking buffer, BB) for 60 min at RT. All antibody incubation steps were performed in BB. Cells were incubated at RT for 45 min using monoclonal anti-DC-SIGN (PN A07406, Beckman Coulter), washed 2 times with PBS, followed by incubation with goat-anti-mouse IgG Alexa 488 (Molecular Probes). After fixation with 1% PFA and permeabilization using 0.1% Triton-X100 in PBS for 5 min at RT, cells were incubated for 45 min with rabbit polyclonal anti-3A(1-60), washed 2 times in PBS, followed by incubation with goat anti-rabbit IgG Alexa 594 (Molecular Probes). After final washes, cells were sealed using Mowiol (Merck) and visualized with a fluorescence microscope.

Blocking studies

Before addition of virus, cells were preincubated at RT for 30 min with isotype control (mouse IgG1), anti-VLA-2 (BD Pharmingen) or a mixture of anti-DC-SIGN mAbs AZN-D1 and AZN-D3 [23, 65] (15 µg/ml). Cells were washed with PBS, resuspended in SF RPMI and infected as described above.

Transfection of viral RNA

p53CB3/T7 and pEV9Hill were linearized by digestion with SalI and NotI, respectively, and transcribed *in vitro* with T7 RNA polymerase. DCs were harvested, washed with PBS and resuspended in phenol-red free Optimem (Invitrogen Life Technologies). RNA (20 µg) was transferred to a 4-mm cuvette to which 200 µl cell suspension containing 3 to 5 X 10^{6} cells was added. Cells and RNA were incubated for 3 min prior to being pulsed using a BioRad GenePulser Xcell (pulse conditions: exponential-wave pulse, 300 V,

150 µF). After transfection, DCs were transferred to full medium and incubated at 37°C. Virus yields were determined at various time points after transfection as described above.

Flow cytometry

After harvest, cells were washed in ice-cold PBA (PBS containing BSA and azide) and added to a v-bottom 96-wells plate. After incubation for 30 min with PBA containing 2% HS, cells were stained using mouse-anti-human monoclonal antibodies against CD40 (mab 89, kind gift of Dr R. de Waal-Malefyt, DNAX Research Institute, Palo Alto, CA), CD80, CD86 (both BD Pharmingen) and CD83 (Immunotech) or the appropriate isotype controls (Pharmingen) on ice for a period of 30 min. Cells were washed twice in PBA and incubated with phycoerythin (PE) labeled goat-anti-mouse IgG (Pharmingen) on ice for a period of 30 min. After washes, cells were fixed in 2% paraformaldehyde and analyzed by flow cytometry on a FACSCalibur apparatus (BD Biosciences). Analysis was done using WinMDI 2.8 software.

ELISA

DC were stimulated with TLR ligands as described above and 75 X 10^3 cells/well were plated out in triplicate in round-bottom 96-wells plates in full medium. After 6 h or 24 h, plates were centrifuged and supernatants harvested and stored at -80°C until cytokine analysis was done. Cytokine concentrations in supernatants were determined using a TNFα or IL-12p70 specific ELISA (both Pierce-Endogen), respectively.

DAPI staining

Immature DC (day 6) were harvested using cold PBS and exposed to CVB3 or EV9 at an MOI of 10 in SF medium. After a 24 h infection period cells were harvested using trypsin-EDTA, resuspended in SF medium and 50 X 10^3 cells were allowed to adhere to poly-L-lysine coated coverslips for 1 h at 37^{o}C. Cells were washed once with PBS and fixed with 1% PFA for 20 min at RT. Following fixation, cells were washed using PBS and incubated at RT using DAPI (5 mg/ml) at a 1:15,000 dilution in PBS for 15 min. After final washes, cells were sealed using Mowiol (Merck) and visualized using a fluorescence microscope.

Caspase-3/7 activity assay

Caspase activity was measured using the Apo-one® homogeneous caspase-3/7 assay kit (Promega) according to manufacturer's instructions. Briefly, caspase-3/7 reagent was prepared by diluting the caspase-3/7 substrate (1:100) with caspase-3/7 buffer. Reagent and cell lysates were added to white 96-well plates (Porvair Sciences), maintaining a 1:1 ratio of reagent to lysate. Enhanced lysis of DCs was achieved by freeze/thawing cells prior to use in assay. Contents were mixed by shaking at 300 rpm on a plate shaker for 2 h at RT. Fluorescence was measured at an excitation wavelength of 485 nm and an emission of 540 nm.

Statistical analysis

The differences in the mean values of cytokine production of infected cells compared to controls were determined by two-tailed Student's *t* test. A P value of <0.05 was considered a significant difference.

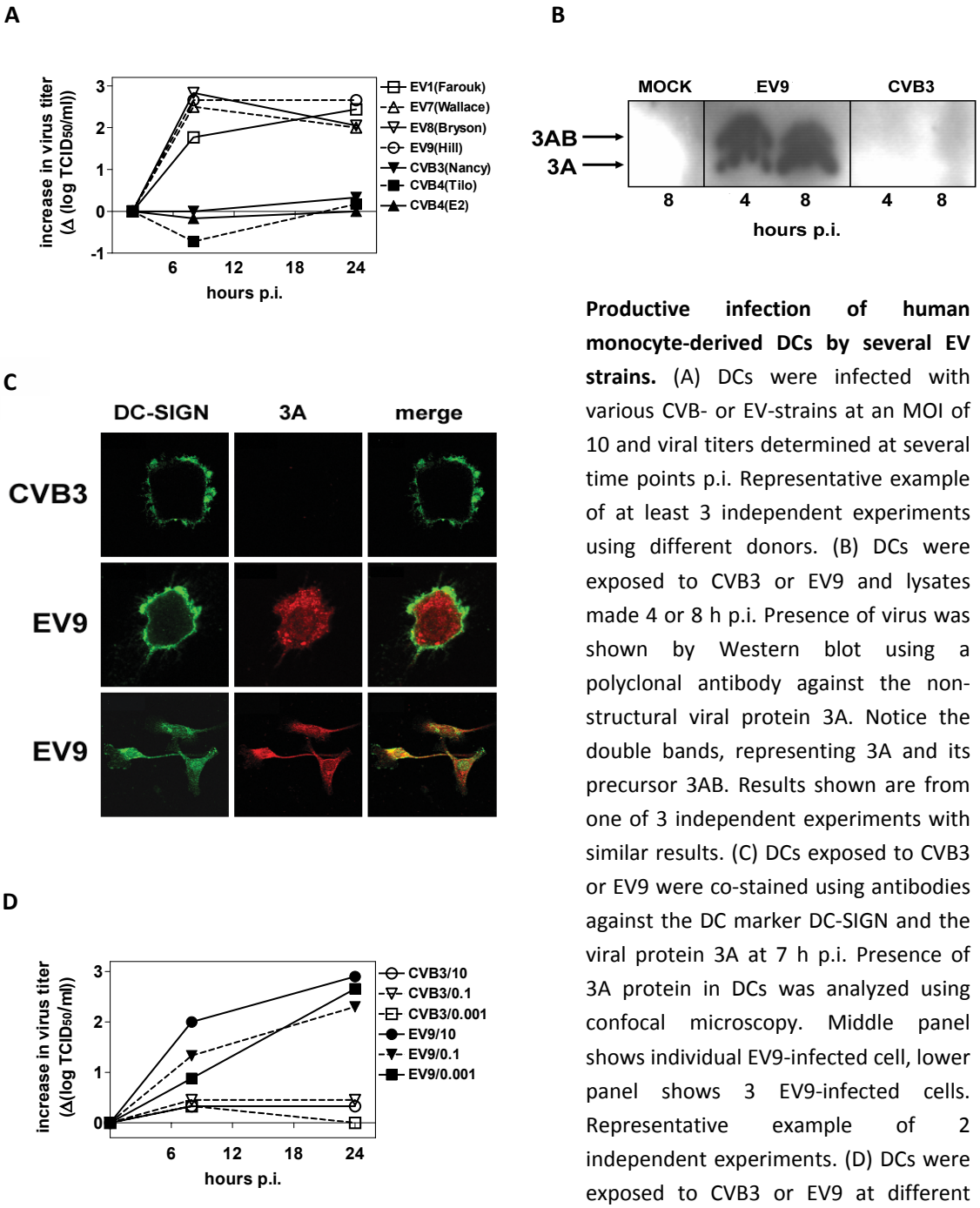
Results

EV, but not CVB, can productively infect human monocyte-derived DCs

To date, little is known concerning the direct effect of infection with CVB or EV on cells of the immune system. We aimed to investigate the capacity of these viruses to infect human DCs and the potential consequences of infection for DC function. Therefore, we selected a number of representatives from the CVB (CVB3 Nancy, CVB4 Tilo and CVB4 Edwards 2) and EV group (EV1 Farouk, EV7 Wallace, EV8 Bryson and EV9 Hill) that are reported to use several different receptors for cell entry. As shown in figure 1A, all EV strains used were capable of infecting DCs, as indicated by a rapid increase in virus titers with maximal virus titers already observed after 8 h. Interestingly, DCs did not support replication of closely related CVB strains, as no increase in the amount of infectious virus was found, even at 48 h post infection (p.i.) (**Fig. 1A and data not shown**). Thus, a clear difference exists in susceptibility of DCs for infection with different HeV-B serotypes. CVB3 Nancy and EV9 Hill (further indicated as CVB3 and EV9) were used during the remainder of our experiments, unless indicated otherwise. Analysis of viral protein synthesis by Western blot using a polyclonal antibody that recognizes the CVB3 and EV9 3A proteins showed the presence of significant amounts of viral protein in DC cultures exposed to EV9, but not CVB3 (**Fig. 1B**). To exclude that viral replication in cells other than DCs accounted for the observed effects, infected cultures were co-stained using antibodies against the viral 3A protein and the DC marker DC-SIGN (*dendritic cell-specific ICAM-3-grabbing nonintegrin*) [23] at 7 h p.i. Immune fluorescence analysis showed that >90% of the EV9-infected cells were positive for both 3A and DC-SIGN. No 3A positive cells could be detected in CVB3-exposed cultures (**Fig. 1C**). This finding confirmed that viral replication takes place in DCs.

To investigate whether infection of DCs at a lower multiplicity of infection (MOI) would also result in efficient replication, the increase in virus titer after exposure of DCs to CVB3 or EV9 at an MOI of 10, 0.1 or 0.001 was determined. Infecting DCs with EV9 at an MOI 10,000 X lower than initially used led to a similar increase in virus titer 24 h p.i., while no replication was observed for CVB3 at any of the MOIs tested (**Fig. 1D**). These results indicate that EV9 is capable of multiple rounds of infection in DC cultures.

Figure 1



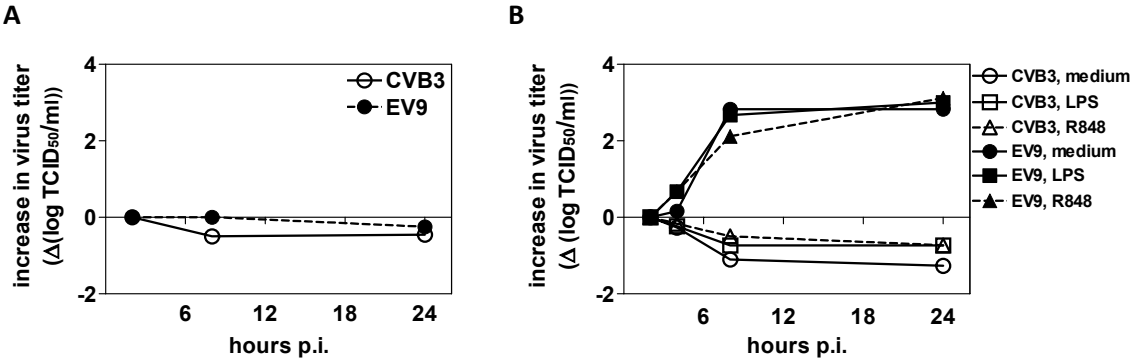
Productive infection of human monocyte-derived DCs by several EV strains. (A) DCs were infected with various CVB- or EV-strains at an MOI of 10 and viral titers determined at several time points p.i. Representative example of at least 3 independent experiments using different donors. (B) DCs were exposed to CVB3 or EV9 and lysates made 4 or 8 h p.i. Presence of virus was shown by Western blot using a polyclonal antibody against the non-structural viral protein 3A. Notice the double bands, representing 3A and its precursor 3AB. Results shown are from one of 3 independent experiments with similar results. (C) DCs exposed to CVB3 or EV9 were co-stained using antibodies against the DC marker DC-SIGN and the viral protein 3A at 7 h p.i. Presence of 3A protein in DCs was analyzed using confocal microscopy. Middle panel shows individual EV9-infected cell, lower panel shows 3 EV9-infected cells. Representative example of 2 independent experiments. (D) DCs were exposed to CVB3 or EV9 at different MOIs and the increase in viral titer was determined at indicated time points.

Mature DCs, but not monocytes, are permissive for EV infection

Since we used monocyte-derived DCs throughout our experiments and taking into account that monocytes can function as DC precursors both *in vitro* and *in vivo* [24-26], we set out to determine whether these cells are also susceptible to infection. Therefore, monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy controls and exposed to CVB3 or EV9. No detectable increase in the amount of infectious virus was observed, indicating that monocytes are not permissive for infection with either of these viruses (**Fig. 2A**). In concordance with these data, no 3A protein could be detected after staining of virus-exposed monocyte cultures with anti-3A antibodies (data not shown).

Because of the apparent difference in susceptibility between (immature) DCs and monocytes, we investigated whether mature DCs would support viral replication. DCs were activated prior to infection by triggering TLR4 or TLR8, involved in recognition of bacteria- or virus-associated structures, respectively. Full DC maturation was achieved, as determined by flowcytometric analysis of the expression levels of maturation markers CD40, CD80, CD83 (data not shown). Infection of both immature and mature DCs with EV9 resulted in an approximate 3-log increase in viral titers over a 24-h infection period, whereas no titer increase could be observed for CVB3 **(Fig. 2B)**. Thus, DCs are highly susceptible to EV infection, irrespective of the DC maturation state.

Figure 2

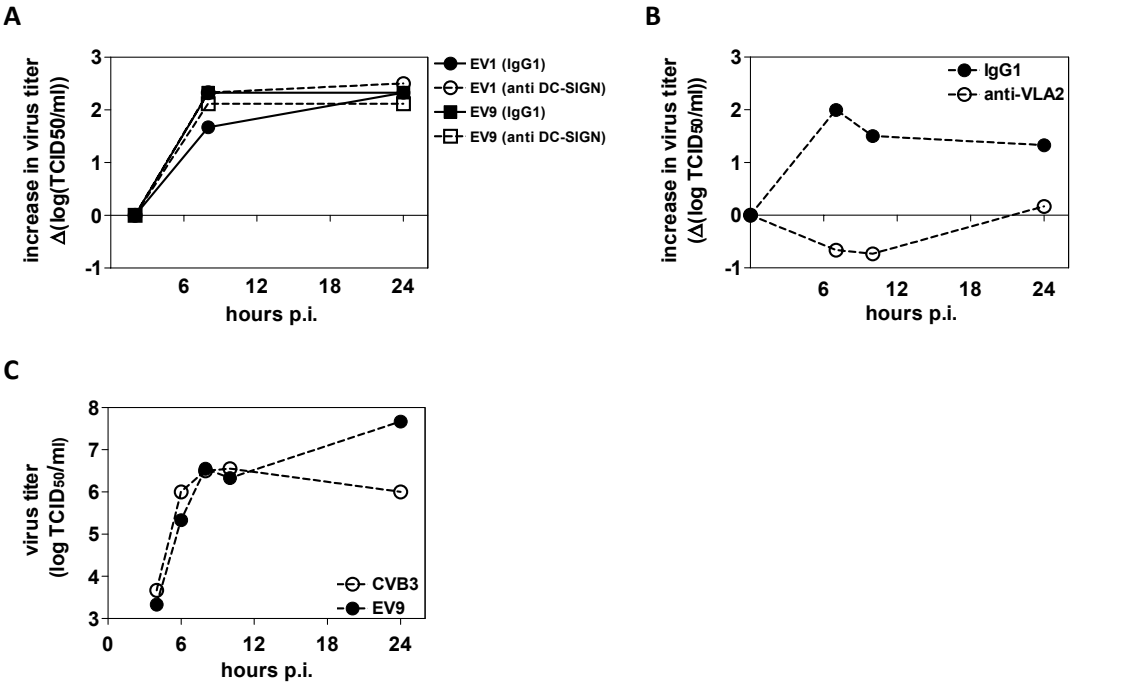


Mature DCs, but not monocytes, are permissive for EV infection. (A) Monocytes were infected as described for DCs in figure 1 and virus titers determined at several time points p.i. (B) Immature DCs were stimulated with TLR ligands LPS (100 ng/ml) or R848 (4 µg/ml) for 24 h or left untreated (medium) and subsequently exposed to CVB3 or EV9 at an MOI of 10, after which viral titers were determined at several time points p.i. Data shown are representative of at least 3 independent experiments.

EV infection of DCs is not mediated via DC-SIGN

DC-SIGN is a C-type lectin specifically expressed by DCs, but not monocytes, and functions as a pattern recognition receptor for many pathogens. In addition, DC-SIGN has been reported to facilitate infection of DCs with several viruses [27, 28]. Considering the difference in susceptibility between monocytes and DCs, we tested whether EV infection of DCs could be mediated through DC-SIGN. Therefore, DCs were pre-incubated with a combination of DC-SIGN blocking antibodies, which have been previously shown to block interaction between DC-SIGN and measles virus [27] as well other pathogens [29, 30]. Blocking DC-SIGN did not affect susceptibility of DCs for either EV9 or EV1 (**Fig. 3A**).

Figure 3



EV infection is not mediated via DC-SIGN, but requires interaction with a specific cell surface receptor. (A) DCs were preincubated with a combination of DC-SIGN blocking antibodies (AZN-D1, AZN-D3, 15 µg/ml) or the relevant isotype controls for a period of 30 min at RT and subsequently infected with EV9 or EV1 at an MOI of 10. Increase in virus titer was determined at several time points p.i. Representative example of 3 independent experiments. (B) DCs were preincubated with a VLA-2 blocking antibody or isotype control for a period of 30 min at RT and subsequently infected with EV1 at an MOI of 10. Increase in virus titer was determined at several time points p.i. Data shown are from one of two representative experiments with similar results. (C) Immature DCs were resuspended in 200 µl phenol-red free Optimem and electroporated with 20 µg *in vitro* transcribed RNA encoding the viral genome of CVB3 or EV9. Viral titers were determined at several time points after transfection. Data shown are representative of 3 independent experiments.

Also pretreatment of DCs with EGTA, a calcium chelator that inhibits C-type lectin function, did not affect infection efficiency (data not shown). These data make it unlikely that DC-SIGN is involved in EV entry into DCs. To date, a number of receptors have been identified for different HeV-B viruses. However, the receptor for EV9(Hill) is yet unknown. The integrin very late antigen-2 (VLA-2) has been recognized as the receptor for EV1 in HeLa cells [31]. To investigate whether infection of DCs is mediated via a specific virus receptor, we tested the effect of VLA-2 blockage on EV1 infection. Blocking of VLA-2 completely abrogated EV1 infection of DCs, whereas an isotype control antibody had no effect (**Fig. 3B**). These data suggest that EV infection of DCs is mediated via a specific virus receptor.

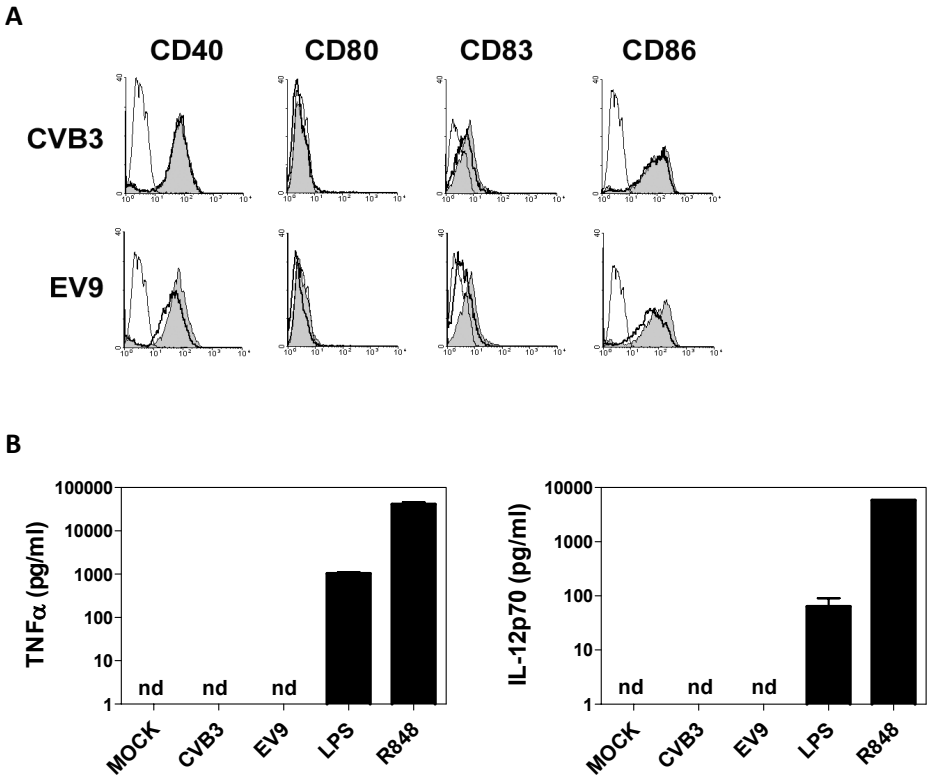
The difference in susceptibility of DCs for CVB and EV could possibly be due to lack of expression of the coxsackievirus and adenovirus receptor (CAR) on DCs. Indeed, no CAR (or *decay accelerating factor* (DAF)) was detected on the cell surface by flow cytometry (data not shown), in concordance with earlier findings [32]. To study whether CVB3 is capable of replicating in DCs upon bypassing its receptor, cells were transfected with RNA transcribed *in vitro* from full-length cDNA clones of CVB3 and EV9. Transfection resulted in rapid virus production and both CVB3 and EV9 reached similar titers at 8 to 10 h after electroporation of viral RNA (**Fig. 3C**). After this time, only the titer of EV9 continued to rise because this virus, but not CVB3, is capable of infecting cells that were not initially transfected. These results support the idea that the incapability of CVB to replicate in DCs is due to the absence of the appropriate receptor.

HeV-B do not induce DC activation

Next, it was studied whether infection leads to DC activation, as determined by analyzing the expression of costimulatory molecules and cytokine production. No upregulation of the maturation-associated molecules CD40, CD80, CD83 and CD86 could be observed after a 24-h infection period (**Fig. 4A**). Instead, EV9 infection led to a small decrease in the expression of some maturation markers, but this effect was not observed consistently. To investigate whether infection of DCs results in the production of pro-inflammatory cytokines, the levels of TNFα and IL-12p70 in DC supernatants were determined at 24 h p.i. No significant levels (<10 pg/ml) of these cytokines were detected after infection, in contrast to the high production observed upon stimulation of DCs with LPS or R848 (**Fig. 4B**). Similar results were found for the production of IL1β, IL-6 and the anti-inflammatory cytokine IL-10 (data not shown).

Viral infection is a potent inducer of type I IFNs in many cell types. However, no IFNα or IFNβ was detected using ELISA and quantitative PCR (qPCR) following infection of DCs with CVB3 or EV9, even at early time points after infection (2, 4, or 6 h p.i.) (data not shown). Preliminary experiments using a new micro fluidic based qPCR technique, to study the expression of a broad array of chemokines and cytokines, revealed that infection did not induce significant changes in any of the target genes analyzed (e.g. IL-6, IL-8, RANTES (*regulated on activation normal T cell expressed and secreted*), CCL4, CCL5 etc) at 7 h p.i. (data not shown). Together, these data indicate that DCs do not become activated upon infection with either EV9 or CVB3.

Figure 4



EV infection does not lead to DC maturation or cytokine production. (A) DCs were exposed to CVB3 or EV9 at an MOI of 10 and the expression of maturation markers CD40, CD80, CD83 and CD86 was determined by flow cytometry 24 h after infection. Thin black lines indicate isotype control. Grey filled histogram represents expression on mock-infected DCs, thick black lines indicate expression levels on CVB- or EV9- exposed DCs. (B) DCs were infected with CVB3 or EV9 and levels of TNFα and IL-12p70 in supernatants of infected DC were determined by ELISA after a 24 h infection period. To determine the capacity of DCs for cytokine production, cells were stimulated in parallel with TLR ligands LPS (100 ng/ml) or R848 (4 μg/ml). Graphs show mean + SD of triplicate measurements. Results shown are representative example of 3 independent experiments.

EV infection impairs TLR responses in DCs

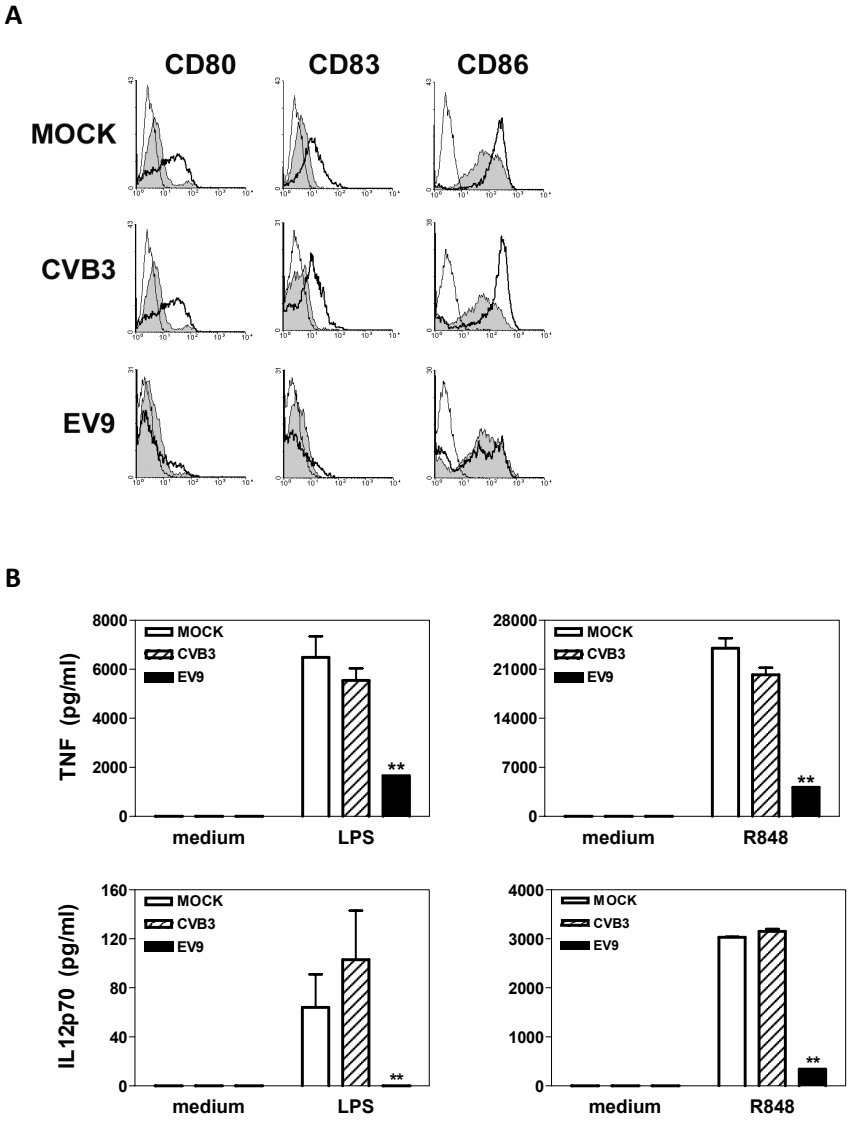
To analyze whether infection affects the response of DCs to TLR ligands, cells were stimulated with LPS or R848 immediately following infection. Both uninfected and CVB3-exposed DCs stimulated with LPS or R848 for 24 h showed an increased expression of CD80, CD83 and CD86 (**Fig. 5A**). In contrast, infection with EV9 prior to TLR stimulation, consistently resulted in a profound reduction of DC maturation. In addition to phenotypic maturation of DCs, TLR ligation leads to the release of cytokines like TNFα and IL-12p70. High levels of these pro-inflammatory cytokines were detected following stimulation with LPS or R848 in supernatants of uninfected and CVB3-exposed DCs. In contrast, EV9-infected DCs produced significantly lower levels of TNFα and this deficiency became apparent as soon as 6 h after stimulation (**Fig. 5B**).

The modest decrease in TNFα production after activation of CVB3-exposed DCs was not observed consistently. IL-12p70 levels were undetectable during these early time points, since it is secreted at a later phase after activation. However, analysis of supernatants at 24 h p.i. showed that the production of this cytokine is also dramatically impaired (**Fig. 5C**). Analysis of cytokine mRNA levels in TLR-activated infected cells revealed that EV9 infection hinders transcription of TNFα and IL-12p70 at very early time points after infection (data not shown). These findings indicate that infection can rapidly shut-down TLR responses in DCs.

Effect of HeV-B on DC viability

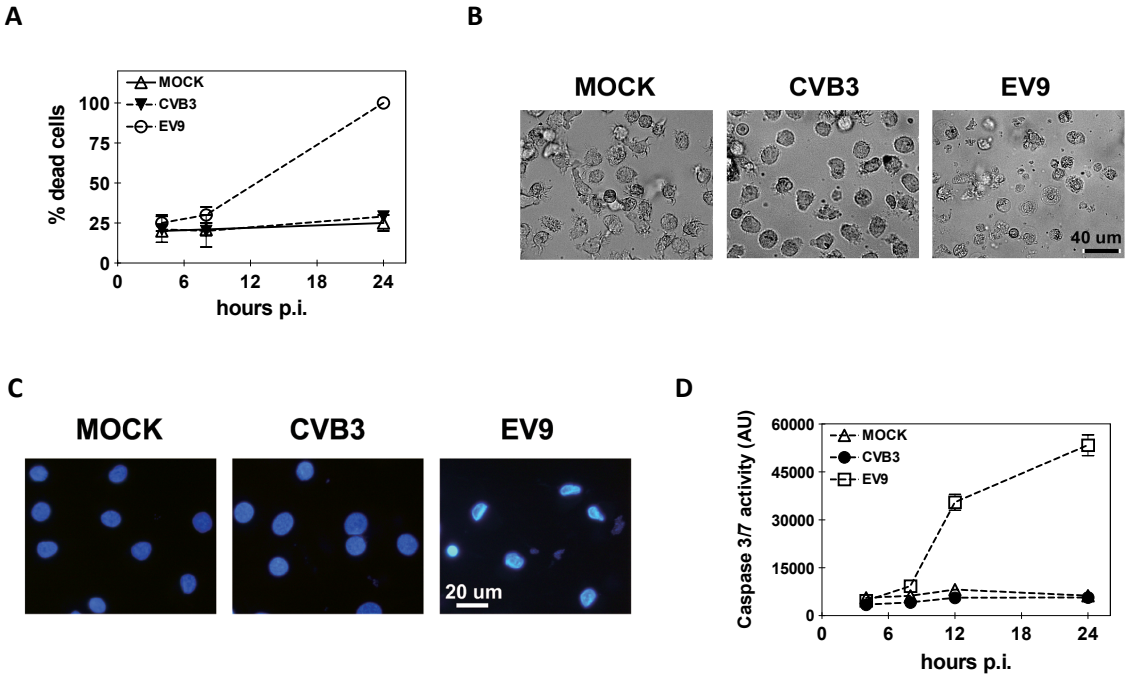
HeV-B virus release from infected cells is generally thought to be mediated via cell lysis. The kinetics of lytic activity was studied in relation to the observed functional impairment of DCs after infection. Therefore, DCs were exposed to CVB3 or EV9 and the percentage of dead cells determined by a Trypan Blue (TB) exclusion assay at different time points p.i. While at 8 h p.i. no significant difference could be observed in cell viability in the different cultures, hardly any viable cells could be detected 24 h after EV9 infection. Survival in mock-infected or CVB3-exposed cultures was comparable (**Fig. 6A**). These findings were confirmed by light microscopic analysis, revealing obvious signs of cell death in EV9-infected DC cultures (**Fig. 6B**). Enteroviruses are known to trigger a complex type of cell death that shows characteristics of both necrosis and apoptosis. Staining of the nuclei of EV9-infected DCs at 24 h p.i. with DAPI revealed predominantly crescent-shaped nuclei with condensed chromatin (**Fig. 6C**), a feature that is typical for enterovirus-infected cells [33]. Only few cells with fragmented nuclei were typically observed. No changes in nuclear phenotype were observed in CVB3-exposed cells.

Figure 5



EV infection impairs responsiveness of DCs to TLR ligands. (A) DCs were exposed to CVB3 or EV9 and immediately stimulated with TLR ligands LPS (100 ng/ml, data not shown) or R848 (4 µg/ml) after last washing step to remove unbound virus. Expression levels of costimulatory molecules CD80, CD83 and CD86 were determined using flow cytometry. Thin black lines represent isotype controls. Grey filled histogram indicate expression on medium stimulated DCs and thick black lines represent expression levels on R848-stimulated DCs. (B and C) DCs were exposed to CVB3 or EV9 and immediately stimulated with LPS or R848. TNFα or IL-12p70 levels in DC supernatants were determined using ELISA after a 6 h or 24 h incubation period, respectively. Graphs show mean + SD of triplicate wells. Representative examples of 3 independent experiments. **, P<0.01 (cytokine production by EV9-infected DCs vs mock-infected or CVB3-exposed DCs; two-tailed Students *t* test).

Figure 6



EV9 infection results in rapid cell death in DC cultures. (A) DCs were exposed to CVB3 or EV9 at an MOI of 10 and the percentage of dead cells in DC cultures was determined at several time points p.i. using a Trypan Blue exclusion assay. Graphs show mean +/- SD of 5 independent experiments. (B) DC morphology 24 h after exposure to CVB3 or EV9, as analyzed using light microscopy. (C) DCs were exposed to CVB3 or EV9 at an MOI of 10 and nuclear staining was performed using DAPI 24 h post exposure. (D) DCs were exposed to CVB3 or EV9 and lysates made at several time points p.i. Caspase-3/7 activity was measured in DC lysates. Graphs show mean +/- SD of triplicate measurements. One representative example of 3 independent experiments is shown.

Analysis of the activity of effector caspases-3 and -7 in infected DCs showed little caspase activity up to 8 h p.i., but a steep increase was observed at later time points in EV9-infected, but not CVB3- exposed cells (**Fig. 6D**). Thus, EV9 infection of DCs triggers rapid cell death that shows signs of both necrosis and apoptosis.

Discussion

In this study, we showed that EV, but not CVB strains, are highly efficient in productively infecting both immature and mature DCs *in vitro*. Remarkably, the infection did not result in activation of DCs. Instead, EV impaired the response of DCs to TLR ligands, since both maturation and cytokine secretion following stimulation with

LPS or R848 were significantly decreased in EV-infected DCs. The clear difference between CVB and EV regarding outcome of infection is most likely related to absence of the appropriate receptor for CVB on the DC surface, since bypassing the receptor by electroporation of CVB RNA resulted in highly efficient replication in DCs. Finally, infection with EV led to rapid killing of DCs within 24 h after infection.

HeV-B belong to the most common viral causes of intestinal infections in life. Although infection usually remains limited to the intestinal tract, the virus can cause serious illnesses by spreading to target organs [12]. In addition, HeV-B infections have been associated with chronic immune disorders [14, 17-19], but direct proof is lacking and a pathogenic mechanism has not yet been elucidated. Explanations are sought either in viral persistence in target organs [19, 34, 35], blood [36-38] or in viral triggering of an autoimmune process [21, 39], theories that are not mutually exclusive. So far, particularly CVB are thought to be involved. However, evidence is growing that other serotypes, like EV, can also play a role [15, 40, 41]. Our study shows that DCs, which are critically involved in orchestration of the immune response, can become directly affected by EV *in vitro*, raising the possibility that EV may modulate immune responses *in vivo*.

In contrast to monocyte-derived DCs and macrophages, monocytes themselves are refractory to HeV-B infection *in vitr*o [42, 43]. In line with this, poliovirus, another member of the genus *Enterovirus*, has recently been shown to replicate in monocyte-derived macrophages but not in monocytes [44]. Thus, within the myeloid lineage, there is a profound difference in susceptibility for infection. Interestingly, it has been shown that also the maturation stage of DCs can determine susceptibility for and outcome of infection with for instance cytomegalovirus [45, 46]. However, we found that both immature and mature DCs were equally supportive of EV replication, whereas neither of them could be productively infected by CVB. Also the type of TLR triggered to induce maturation (*e.g.* TLRs involved in recognition of bacterial vs viral structures) could possibly affect outcome of infection. Therefore, we determined susceptibility of DCs which had been activated with LPS (TLR4) or R848 (TLR7/8). Both ways of DC activation allowed for similar replication efficiency of EV in DCs. These findings indicate that DCs are highly permissive for infection with EV, independent of DC maturation stage or nature of the maturation stimulus. Also poliovirus has recently been shown to productively infect both immature and mature DCs, although it should be noted that in their study the DCs were not activated using TLR ligands [44].

The difference in susceptibility between monocytes and DCs, led us to investigate whether infection was mediated by DC-SIGN, a DC-specific C-type lectin that was discovered in our lab and is crucially involved in recognition and uptake of many different pathogens [47]. It has recently been described that DC-SIGN can serve as a receptor for measles virus [48] and human herpesvirus 8 [28], thereby facilitating infection of DCs. Blocking of DC-SIGN did not alter the susceptibility of DCs for EV infection. However, infection with EV1 could be prevented by blocking VLA-2, the EV1 receptor. These data suggest that EV infection most likely takes place through interaction with a specific cell surface receptor rather than DC-SIGN. In concordance with this idea, no CAR or DAF expression was found on DCs, which explains the incapability of the CVB strains tested to infect DCs. CVB3 was capable of efficient replication upon transfection of viral RNA. The latter finding suggests that DCs, when infected via alternative routes, may also become functionally affected by CVB. Such alternative routes can be antibody-mediated uptake via Fc-receptors, or phagocytosis of infected, dying cells. Indeed, it has recently been described that the susceptibility of PBMC and monocytes to CVB4 can be enhanced via an antibody-dependent mechanism [42, 49]. This mechanism might provide a possible explanation for the observed presence of CVB in PBMCs of some T1D patients at onset of the disease [50]. Whether such alternative routes mediate infection of DCs by CVB requires further investigation.

Although several viruses induce DC activation upon infection [51, 52] and exposure of monocytes or PBMC to HeV-B results in the production of cytokines [53-55], infection of DCs with CVB or EV did not result in DC activation. This unresponsiveness is striking since DCs play a central role in the defense against viruses and other microorganisms. However, enteroviruses are known to deploy several strategies to curtail anti-viral host cell responses. This is achieved by for instance the action of viral proteases that cleave transcription factors and translation initiation factors, thereby shutting off cellular gene expression [56, 57]. In addition, the 3A protein has been shown to inhibit ER-to-Golgi transport [58, 59] thereby interfering with cytokine secretion and antigen presentation [60, 61]. These mechanisms might underlie the absent upregulation of co-stimulatory molecules and lack of synthesis of cytokines like type I IFNs, TNFα and IL-12 in infected DCs. In addition, the virus affected the response of DCs to TLR ligands. This might be mediated via above mentioned effects of infection on gene expression or protein transport or altered TLR expression levels or localization. Furthermore, decreased cell viability following infection might also contribute to inhibited TLR responses. However, we found that also early responses, such as TNFα production, were dramatically impaired, already before signs of cell death became apparent. This

finding demonstrates that the EV not only suppresses DC activation as a direct result of infection but also limits TLR-mediated immune responses. Previous studies have yielded similar results upon infection of DCs with other viruses [9, 62]. Our findings add EV to the growing list of pathogens that can interfere with DC function.

In summary, our data demonstrate for the first time that DCs can become productively infected with EV, but not with CVB strains. Infection does not result in DC activation or the induction of anti-viral immune responses, but instead impairs the response to TLR ligands and induces cell death. This might represent an important evasion strategy, as targeting DCs would interfere with stimulation of T cells specific for EV or other pathogens. Consequently, failure in clearing virus-infected cells could result in persistent infection and possibly contribute to HeV-B associated diseases, such as T1D and chronic myocarditis.

Acknowledgments

This work was supported by grants from the Netherlands Diabetes Foundation (DFN 2001.00.047) to M.K. and The Netherlands Organization for Scientific Research (NWO-VIDI-917.46.305) to F.J.M.v.K. and (NWO-912-02-034) to G.J.A.

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Chapter 7

Phagocytosis of picornavirus-infected cells induces an RNA-dependent antiviral state in human dendritic cells

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Journal of Virology (2008) 82(6): 2930-2937

Abstract

Dendritic cells (DCs) play a central role in instructing antiviral immune responses. DCs, however, can become targeted by different viruses themselves. We recently demonstrated that human DCs can be productively infected with echoviruses (EV), but not coxsackieviruses (CVB), both of which are RNA viruses belonging to the Enterovirus genus of the Picornavirus family. We now show that phagocytosis of CVB-infected, type I IFN deficient cells induces an antiviral state in human DCs. Uptake of infected cells increased the expression of the cytoplasmic RNA helicases RIG-I and MDA5 as well as other *interferon stimulated genes* and protected DCs against subsequent infection with EV9. These effects depended on recognition of viral RNA and could be mimicked by exposure to the synthetic dsRNA analogue poly(I:C), but not other TLR-ligands. Blocking endosomal acidification abrogated protection, suggesting a role for TLRs in the acquisition of an antiviral state in DCs. In conclusion, recognition of viral RNA rapidly induces an antiviral state in human DCs. This might provide a mechanism by which DCs protect themselves against viruses when attracted to an environment with ongoing infection.

Introduction

Dendritic cells (DCs) are crucial for the induction of antiviral immunity via instruction of both the innate and adaptive immune system [1-3]. The hallmark of antiviral responses is production of type I IFNs. Although classically plasmacytoid DCs were thought to be the major producers of type I IFNs [4, 5], it has been shown that conventional DCs can produce similarly high type I IFN levels upon viral infection [6]. In fact, all nucleated cells are capable of type I IFN production, resulting in the establishment of an antiviral state in neighboring cells via upregulation of so-called *interferon stimulated genes* (ISGs).

DCs express a vast array of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) that enable them to recognize viral pathogen-associated molecular patterns (PAMPs) like dsRNA [7] and ssRNA [8-10]. TLRs responding to viral nucleic acids are localized within the endosomal compartment [11, 12] and are triggered upon endocytosis of viral particles or virus-infected cells, but presumably do not sense cytoplasmic virus replication upon infection of the DC itself. Instead, recent studies have identified two structurally related RNA helicases, *retinoic acid inducible gene I* (RIG-I) [13] and *melanoma differentiation associated gene 5* (MDA5) [14], both ISGs, as critical mediators in the response to infection with different RNA viruses in mice [15-17]. Triggering of MDA5 or RIG-I causes recruitment of the shared adaptor protein *IFN-β promoter stimulator 1* (IPS-1), also known as *mitochondrial antiviral signaling protein* (MAVS), *virus-induced signaling adaptor* (VISA), and *CARD adaptor inducing IFN-β* (Cardif) [18-21], leading to induction of type I IFNs. RIG-I deficient cells display greatly diminished type I IFN responses to various RNA viruses and *in vitro* transcribed dsRNA [17, 22]. Interestingly, MDA5 deficient cells are selectively unresponsive to certain picornaviruses, such as encephalomyocarditis virus (EMCV) and Theiler's encephalomyelitis virus as well as the synthetic dsRNA analogue poly(I:C) [15, 17]. This specificity might be related to the presence or absence of 5' triphosphate groups, which was recently reported to be a critical structure for RIG-I activation [23, 24]. The essential contribution of these RNA helicases to antiviral immunity becomes evident in RIG-I or MDA5 KO mice that readily succumb upon infection with Japanese encephalitus virus or EMCV, respectively [15, 17].

To date, most data regarding RIG-I and MDA5 are derived from mouse studies, while still little is known regarding the role of these viral sensors in human cells. We recently showed that infection of human monocyte-derived DCs with EV, but not CVB, results in

rapid inhibition of TLR-mediated responses and massive cell death [25]. As it is difficult to reconcile these *in vitro* effects with the generally mild clinical outcome of EV infections, we set out to investigate the conditions that could potentially alter DC susceptibility. In the present work, we demonstrate that phagocytosis of virus-infected cells results in enhanced expression of RIG-I, MDA5 and other ISGs and protects DCs against EV infection. These effects require intact endosomal acification and depend on the presence of RNA in infected cells. Thus, DCs engage a state of antiviral resistance following recognition of viral RNA.

Materials & Methods

Virus stocks and purification

Reference strains Echovirus 1 Farouk (EV1 Farouk), EV7 Wallace, EV8 Bryson, EV9 Hill and EV11 Gregory were obtained from the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). CVB3 Nancy was kindly provided by R. Kandolf (University of Tübingen, Germany). Production of virus stocks and virus titrations were performed on buffalo green monkey cells as described previously [25]. Serial 10-fold dilutions were tested in 96-well microtiter plates and fifty percent Tissue Culture Infective Doses ($TCID_{50}$) were calculated as described before [26].

Plasmids

The EV9 Hill infectious cDNA clone [27] was generously provided by B. Nelsen-Salz (Virology Institute, University of Cologne, Germany).

Stimulation of monocyte-derived DCs

Monocyte-derived DCs were generated as described previously [25]. Mature DCs were obtained by stimulating cells with poly(I:C) (20 μg/ml), LPS (100 ng/ml), R848 (4 μg/ml), PAM_3Cys-SKKKK (PAM, 2 μg/ml) for a period of 24 h, unless indicated otherwise. To block the actions of type I IFN, cells were stimulated in presence or absence of neutralizing anti-human type I IFN antibodies (1:100, Iivari, Kaaleppi or bovine serum, courtesy of professor Julkunen, National Public Health Institute, Helsinki, Finland)[28]. To block endosomal acidification, DCs were cultured with chloroquine (CQ, 10 μM) starting 1 h prior to poly(I:C) stimulation. For infection, immature or mature DCs were harvested using cold PBS, washed and infected at an MOI of 1 with indicated viruses in SF RPMI. After 60 min incubation at 37^{o}C, cells were washed 3 times in an excess volume of PBS after which viral titers were determined at different time points post infection (p.i.) as described above.

Western blot

Equal amounts of protein were separated by 12.5% SDS-PAGE, electroblotted onto nitro-cellulose membranes (Bio-Rad), followed by probing with the indicated antibodies. Anti-RIG-I and anti-PKR antibodies were purchased from ProSci Incorporated and Becton Dickinson Transduction Laboratories, respectively. Production of rabbit polyclonal anti-MDA5 was described previously [29]. RIG-I, PKR and MDA5 antibodies were used in 1:1,000; 1:500 and 1:10,000 dilutions, respectively. After washes, membranes were incubated with IRDye anti-mouse or anti-rabbit IgG (1:15,000) (Li-Cor Biosciences). Imaging was done using the Odyssey System. Western blot analysis of viral protein 3A was done as described before [25].

Transfection of viral RNA

pEV9Hill was linearized by digestion with NotI and transcribed *in vitro* with T7 RNA polymerase (Promega). DCs were harvested, washed with PBS and resuspended in phenol-red free Optimem (Invitrogen Life Technologies). RNA electroporation was performed as described before [25].

RNA isolation

Total RNA was isolated from DC cultures using TRIZOL reagent (Invitrogen Life Technologies) according to manufacturer's instructions, with minor modifications. RNA integrity was determined by analyzing the ribosomal 28S and 18S bands on a 1% agarose gel. The reverse transcription reaction was performed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. For each sample a "-RT" control was included in which the reverse transcriptase was replaced by DEPC treated milli-Q. The cDNA was stored at -20°C until further use.

Quantitative PCR

Quantitative analysis of gene expression in DCs was done using SYBR Green based qPCR. The qPCR reactions were performed in a 25 µl volume containing: 12.5 µl SYBR Green mix (Applied Biosystems), 1.5 µl forward/reverse primer (300 nM endconcentration), 4.5 µl milli-Q and 5 µl cDNA dilution. Reactions were performed on an ABI 7900HT Sequence Detection System (Applied Biosystems). Analysis was done using sequence detection system software (SDS, version 2.0, Applied Biosystems). Primer sequences are available upon request and were designed using the freely accessible Primer Bank program [30].

Uptake of Vero cells

Vero cells were labeled using PKH26 (Sigma-Aldrich) according to manufacturers instructions and infected with CVB3 at an MOI of 10. Cells were harvested and washed 6-8 h p.i. and resuspended in fresh medium at a density of 5 X 10^6 cells/mL prior to placing them at -20°C until further use. Vero cell preparations were added to DC cultures at a ratio of 1:1 (in some experiments DCs had been pretreated with CQ as described above). Alternatively, Vero cell preparations were exposed to a mixture of RNAse A (Roche) and RNAse V-I (Ambion) or an equal volume of PBS for a period of 15 min at 37°C prior to addition to DCs. Uptake of Vero cells by DCs was analyzed using flowcytometry and confocal microscopy.

Confocal microscopy

DCs were harvested, washed and allowed to adhere to poly-L-lysine coated coverslips in serum free medium for 1 h at 37^{o}C. Cells were fixed with 1% paraformaldehyde (PFA) and blocked in PBS with 3% BSA, 10 mM glycine and 2% human serum (blocking buffer, BB). For cell surface stainings, cells were incubated using mouse-anti-human DC-SIGN (Beckman Coulter) or mouse-anti human HLA-DR/DP (ascites) in BB, washed and incubated with isotype specific Alexa labeled goat-anti-mouse IgGs (Alexa 568/Alexa 647, Molecular Probes). For intracellular staining, cells were fixed using 1% PFA, permeabilized using 0.1% Triton-X100 in PBS and incubated with rabbit polyclonal anti-MDA5 followed by incubation with goat anti-rabbit IgG Alexa 488 (Molecular Probes) in BB. After final washes, cells were sealed using Mowiol (Merck) and analyzed using confocal microscopy (Biorad MRC 1024).

Statistical analysis

Statistical analysis was performed using Student's *t* test (2-tailed distribution). A P-value < 0.05 was considered a significant difference.

Results

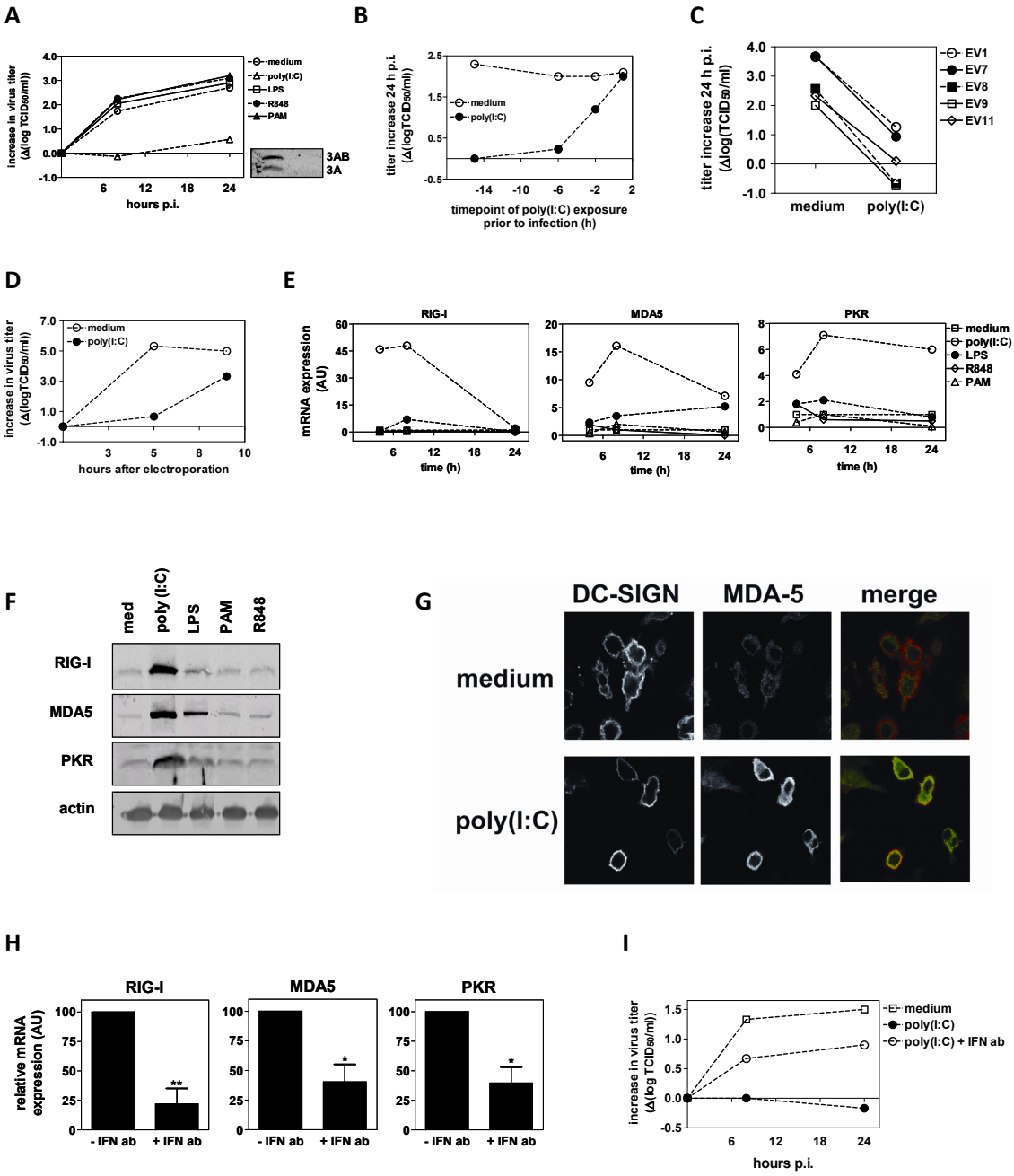
Poly(I:C) induces resistance against viral infection in human DCs

We previously reported that human DCs display a striking difference in susceptibility to infection with distinct enteroviruses, a genus of the picornavirus family. While DCs are productively infected with EV, they are resistant to infection with the closely related CVB[25]. Here, we investigated the effect of stimulation with different TLR ligands on susceptibility for EV infection. Untreated DCs and DCs stimulated with LPS (TLR4), PAM_3Cys (TLR1/2) or R848 (TLR7 or TLR8) were all highly susceptible to EV9 infection, leading to significant increases in virus titers and viral proteins (**Fig. 1A**). In contrast, stimulation with the synthetic viral dsRNA analogue poly(I:C) dramatically reduced EV9 replication (**Fig. 1A**), in a concentration dependent manner (data not shown). Time course analysis showed that resistance to EV9 infection was accrued within 6 h after poly(I:C) exposure (**Fig. 1B**). Additionally, infection of DCs with EV1, EV7, EV8 and EV11 could also be inhibited by poly(I:C) treatment, showing that the protective effect applied to a broad range of EV strains (**Fig. 1C**). Poly(I:C) stimulated DCs also displayed reduced virus replication upon delivery of *in vitro* transcribed EV9 RNA directly into the DC cytoplasm, suggesting the induction of an active antiviral state, rather than simple downregulation of the (as yet unidentified) EV9 receptor on the cell surface (**Fig. 1D**).

To evaluate the effects of TLR triggering on expression of genes that are crucially involved in the innate antiviral response, we determined mRNA levels of the viral sensors RIG-I and MDA5 and the effector molecule PKR. Stimulation with PAM, R848 or lipoteichoic acid (LTA) did not affect ISG expression and only a modest increase was observed in some experiments using LPS. However, stimulation with poly(I:C) consistently induced a strong upregulation of RIG-I, MDA5 and PKR (**Fig. 1E** and data not shown). Western blot (WB) analysis corroborated our findings by qPCR and demonstrated elevated protein levels following exposure to poly(I:C) (**Fig. 1F**). Confocal analysis showed an increased expression of endogenous MDA5 in the cytoplasm of poly(I:C) stimulated human DCs (**Fig. 1G**). Furthermore, neutralizing antibodies against type I IFN inhibited the effect of poly(I:C) on both expression of ISGs (**Fig. 1H**) and viral infection (**Fig. 1I**) implying that poly(I:C) exerts its effect at least in part via autocrine type I IFN stimulation.

Collectively, these data indicate that exposure to synthetic dsRNA causes upregulation of both viral sensors and effector molecules and protects DCs against EV infection.

Figure 1



Poly(I:C) stimulation protects human DCs against EV infection and increases expression of RIG-I, MDA5 and PKR. (A) DCs were left untreated or stimulated with poly(I:C) (20 µg/ml), LPS (100 ng/ml), R848 (4 µg/ml) or PAM_3Cys-SKKKK (PAM, 2 µg/ml) for 24 h and infected with EV9 at an MOI of 1, after which viral titers were determined at several time points p.i. Insert shows the expression of viral protein 3A and its precursor 3AB in unstimulated DCs (left lane) or poly(I:C) stimulated DCs (right lane). (Figure legend continues on next page).

(B) DC were stimulated with poly(I:C) (20 μg/ml) at different time points prior to infection with EV9 at an MOI of 1. Increase in viral titers was determined 24 h p.i. (C) DCs were left untreated or stimulated with poly(I:C) (20 μg/ml) for a period of 24 h and subsequently infected with EV1, EV7, EV8, EV9 or EV11 at an MOI of 1. Shown is the increase in viral titers 24 h p.i. (D) DCs were stimulated with poly(I:C) (20 μg/ml) for 24 h and subsequently electroporated using 20 μg *in vitro* transcribed RNA from the full-length cDNA clone of EV9. Increase in viral titers was determined at several time points after electroporation. (E) DCs were stimulated using LPS, R848, PAM or poly(I:C) as described for (A) and mRNA levels of RIG-I, MDA5 and PKR were determined using qPCR at several time points after stimulation. (F) Protein expression of RIG-I, MDA5 and PKR was analyzed by western blot 24 h after stimulation of DCs as described in (A). (G) DCs were left untreated or stimulated with poly(I:C) (20 μg/ml) for 24 h after which DCs were harvested, stained using DC-SIGN and MDA5-specific antibodies and analyzed using confocal microscopy as described in materials and methods. (H) DCs were stimulated with poly(I:C) (20 μg/ml) in the presence or absence of type I IFN neutralizing antibodies (Iivari, Kaaleppi and bovine anti-IFNα, see materials and methods). After 8 h, mRNA expression levels of RIG-I, MDA5 and PKR were determined using qPCR. Shown is mean expression + SD of 3 independent experiments using different donors (*, P≤0.05; **, P≤0.01). (I) DCs were stimulated with poly(I:C) (20 μg/ml) in the presence or absence of type I IFN neutralizing antibodies for 24 h, washed and subsequently infected with EV9 at an MOI of 1 after which virus titers were determined at several time points p.i. Data shown is representative of >5 (A, E), 3 (C, D, F, G, H, I)) or 2 (B) independent experiments

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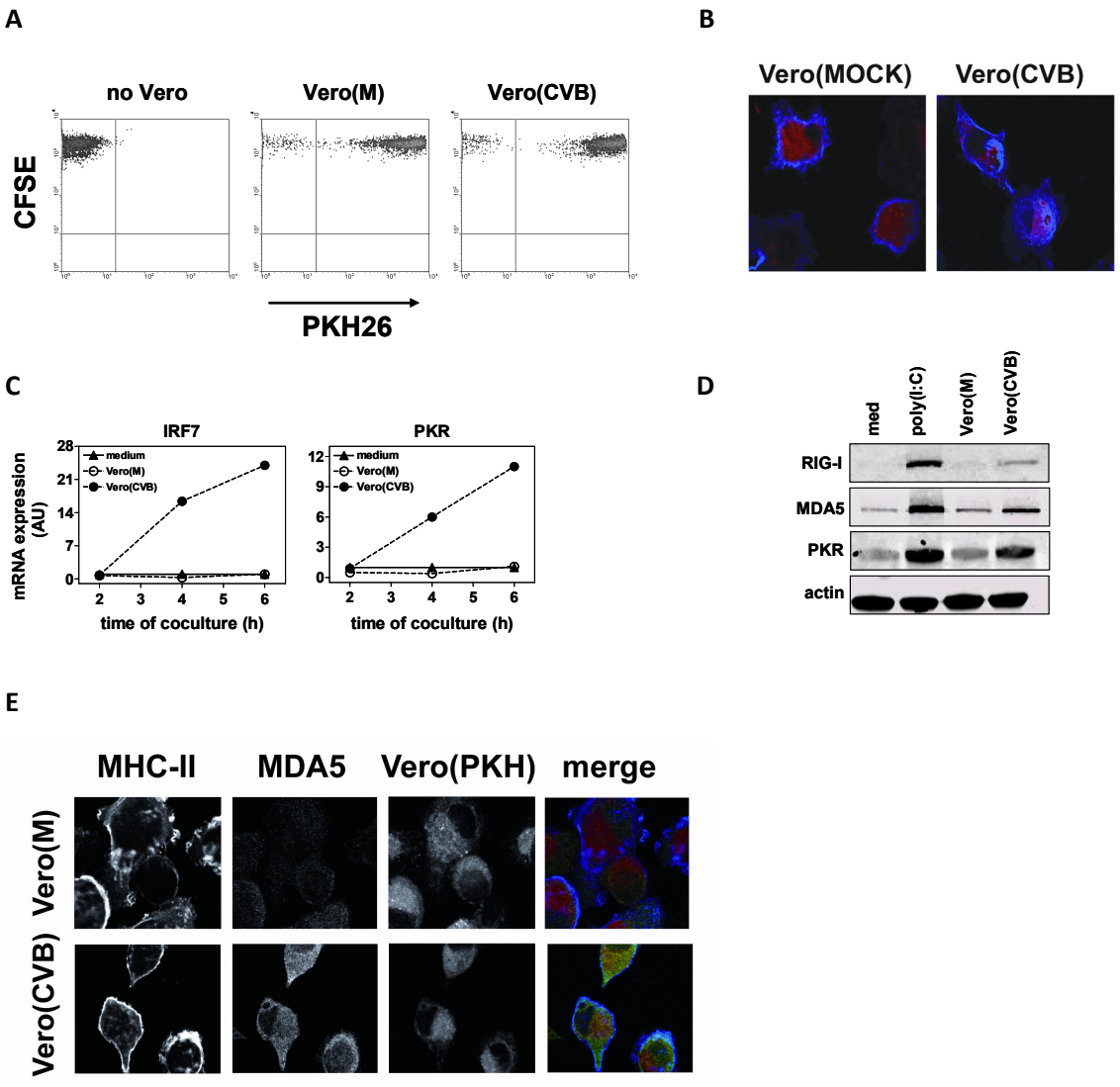
Phagocytosis of infected cells by DCs leads to upregulation of ISGs

To study the DC-virus interaction in a more physiological model, we explored whether phagocytosis of CVB3-infected cells by DCs would induce similar effects as stimulation with poly(I:C). CVB3 was used because this virus, if released from infected (dying) cells, does not cause infection of DCs [25] nor upregulation of RIG-I, MDA5 or PKR (data not shown). Since secretion of type I IFNs by infected cells could influence susceptibility for infection and ISG expression in DCs, all experiments were performed with Vero cells, which carry a genetic defect in type I IFNs synthesis [31, 32]. CVB3-infected, but not uninfected Vero cells, were efficiently taken up by DCs (data not shown), likely reflecting the effect of infection on Vero cell viability. For the remainder of our experiments we used freeze-thawed cell preparations that were taken up with equal efficiency, irrespective of the infection status of the cells (**Fig. 2A)**. This enabled us to analyze the effect of uptake of uninfected or virus-infected cells on ISG expression and susceptibility in DCs. Confocal analysis confirmed the flowcytometry data by showing the presence of $PKH26^{+}$ compartments within DCs (**Fig. 2B**). As the primers for human RIG-I and MDA5 cross-react with their Vero cell (*Cercopithecus aethiops*, African green monkey) homologues, we focused on mRNA expression of the ISGs IRF7 and PKR.

Figure 2



Phagocytosis of virus-infected cells increases the expression of RIG-I, MDA5 and PKR (A) PKH26 labeled Vero cell preparations (mock-or CVB3-infected) were added to CFSE labeled DC cultures in a ratio of 1:1. After 24 h, the number of $CFSE^{+}PKH26^{+}$ DCs was analyzed using flowcytometry. (B) Presence of $PKH26^{+}$ Vero cell material within DCs after a 24 h coculture as analyzed using confocal microscopy. DCs were stained using anti-MHCII (C) DCs were cocultured with mock- or CVB3-infected Vero cell preparations and the mRNA expression of PKR and IRF7 was determined using qPCR at different time points after start of coculture. (D) DCs were cocultured with Vero cell preparations as described in (A) and protein expression of RIG-I, MDA5 and PKR was determined using western blot 24 h after start of coculture. (E) DCs were cocultured with Vero cell preparations as described in (A) and 24 h later cells were stained with MHC-II and MDA5-specific antibodies and analyzed using confocal microscopy. Data shown are representative of >6 (A), 4 (B) or 3 (C-E) independent experiments using different donors.

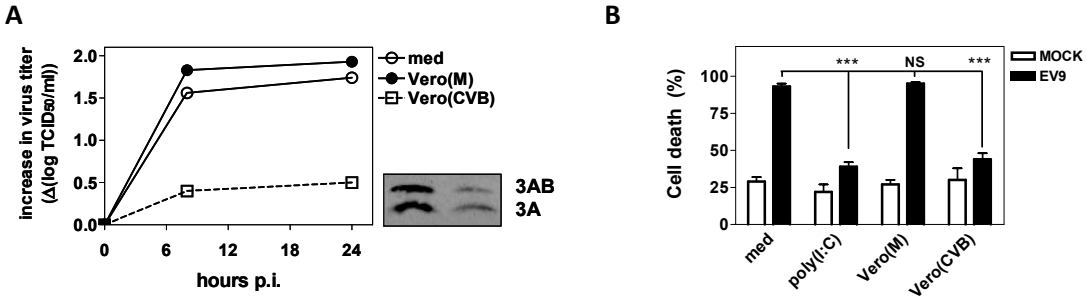
Although uptake of mock-infected Vero cells had no effect, phagocytosis of CVB3-infected cells caused a rapid and profound upregulation of IRF7 and PKR in DCs (**Fig. 2C**). Western blot analysis showed that uptake of CVB-infected, but not of mock-infected Vero cells, resulted in increased protein levels of RIG-I, MDA5 and PKR (**Fig. 2D**). No ISG protein expression was detected in CVB3-infected Vero cell preparations as such (data not shown). Confocal analysis further confirmed increased MDA5 levels in DCs that had taken up CVB3-infected Vero cells as compared to mock-infected cells (**Fig. 2E**). Thus, phagocytosis of CVB3-infected cells by DCs leads to rapid upregulation of molecules involved in the innate antiviral response and can occur independently of type I IFN released by infected cells.

Phagocytosis of CVB-infected cells protects DCs against EV infection

To determine whether upregulation of ISGs following uptake of infected cell preparations by DCs resulted in functional protection, DCs were subsequently infected with EV9. Vero cell preparations themselves did not support replication of EV9 (data not shown). Phagocytosis of infected preparations resulted in a markedly decreased EV replication in DC cultures, while uptake of uninfected cells had no effect (**Fig. 3A**). Western blot analysis showed notably lower levels of the viral protein 3A and its precursor 3AB in DCs that had taken up CVB-infected Vero cells, which confirmed inhibited EV growth (**Fig. 3A**).

We next assessed whether phagocytosis of these preparations could protect DCs against EV9-induced cell death. As shown in **figure 3B**, EV9 caused massive cell death in both untreated DCs and DCs that had taken up uninfected cell preparations. In contrast, uptake of CVB3-infected cell preparations strongly enhanced cell survival. Also exposure to poly(I:C) decreased cell death following EV infection. Taken together, these data indicate that phagocytosis of virus-infected cells can effectively protect human DCs against the lethal effects of EV infection.

Figure 3



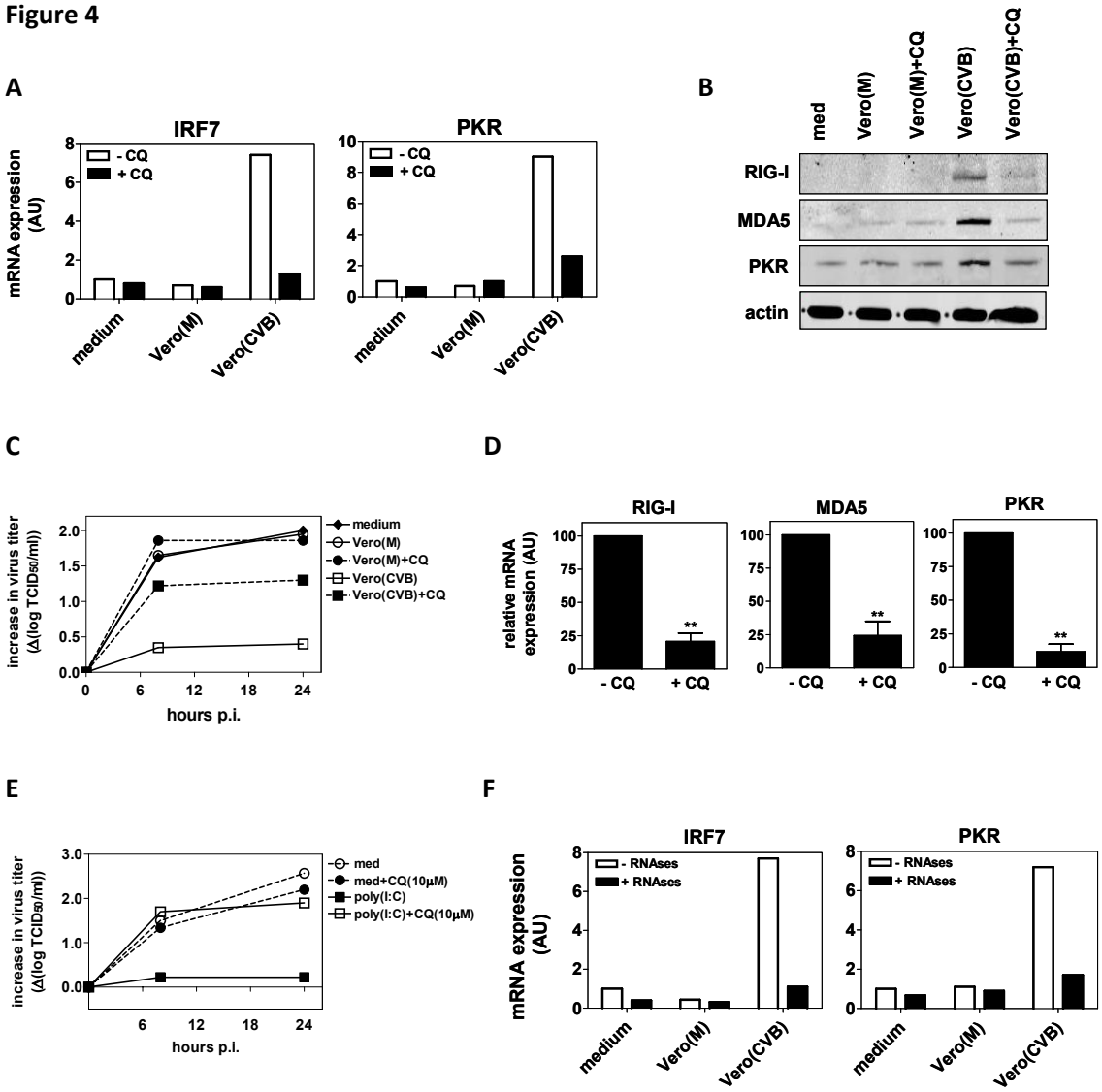
Phagocytosis of infected cells protects DCs against EV infection. (A) DCs were left untreated or cultured with mock or CVB-infected Vero cell preparations for a period of 24 h after which DCs were harvested, washed and subsequently infected with EV9 at an MOI of 1. Viral titers were determined at different time points p.i. Insert shows the expression of viral protein 3A and its precursor 3AB following uptake of mock-infected (left lane) or CVB3-infected (right lane) Vero cells, 8 h after infection of DCs with EV9. (B) DCs were treated as described in (A) and the percentage of dead cells was determined using a trypan blue exclusion assay 48 h p.i. Graph shows mean + SD of 4 quadruplicates per condition (***, $P \leq 0.001$). Data shown are representative of at least 3 independent experiments using different donors.

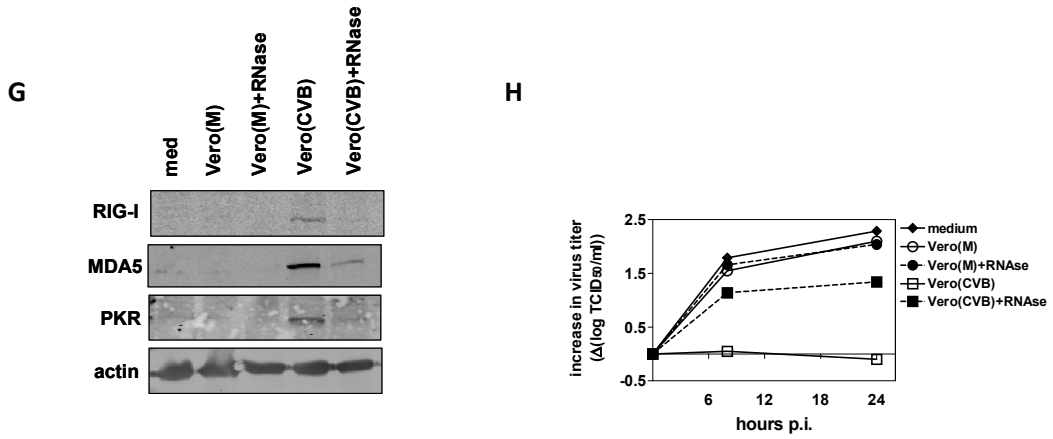
ISG upregulation in DCs following phagocytosis of infected cells requires intact endosomal acidification and is mediated via recognition of viral RNA

Infected -dying- cells taken up by DCs are localized to so-called phagosomes, which subsequently "mature" via fusion with the endosomal/lysosomal compartments, resulting in a progressive decrease in pH [33]. Interestingly, intracellular TLRs are also recruited to these compartments, enabling interaction with potentially released PAMPs, like dsRNA, which occurs in a pH dependent fashion[34]. To determine if endosomal acidification is required for the induction of viral resistance following uptake of infected cell preparations, we pretreated DCs with chloroquine (CQ), a chemical that blocks acidification of these compartments. Pretreatment of DCs with CQ markedly decreased mRNA expression of IRF7 and PKR (**Fig. 4A**) as well as RIG-I, MDA5 and PKR protein levels (**Fig. 4B**). Importantly, the CQ mediated reduction in ISG levels was accompanied by reduced protection against EV infection (**Fig. 4C**). These effects were not related to impaired phagocytosis, since CQ had no effect on uptake of Vero cell preparations (data not shown). CQ also reduced the poly(I:C) mediated increase in ISG expression (**Fig. 4D**) and completely abrogated the inhibitory effect of poly(I:C) on infection (**Fig. 4E**).

We next investigated the potential contribution of viral RNA present in the infected Vero cell preparations to both upregulation of ISGs and the induction of a resistant state. To this aim, CVB3-infected and uninfected cell preparations were incubated with a mixture of RNAses prior to addition to DCs. RNAse treatment did not affect uptake of preparations by DCs (data not shown). Degradation of RNA resulted in a strongly reduced upregulation of IRF7 and PKR mRNA levels (**Fig. 4F**) and RIG-I, MDA5 and PKR protein expression in DCs (**Fig. 4G**).

Figure 4





Induction of an antiviral state requires intact endosomal acidification and is mediated via recognition of viral RNA. (A) DC were left untreated or preincubated with CQ (10 µM) for 1 h prior to addition of Vero cell preparations. After an 8 h coculture, cells were harvested and mRNA expression of IRF7 and PKR was analyzed using qPCR. (B) DCs were stimulated as in (A) and protein expression of RIG-I, MDA5 and PKR was determined using WB 16 h after coculture (C) DCs were stimulated as in (A) and infected with EV9 at an MOI of 1 16 h after coculture. Viral titers were determined at several time points p.i. (D) DC were left untreated or preincubated with CQ (10 µM) prior to addition of poly(I:C) (20 µg/ml). After 8 h stimulation, the expression of RIG-I, MDA5 and PKR and IRF 7 was analyzed using qPCR. Shown is mean expression + SD of 3 independent experiments using different donors (**, P≤0.01). (E) DCs were treated as described in (A) and after 8 h stimulation, cells were harvested, washed extensively and infected with EV9 at an MOI of 1, after which viral titers were determined at several timepoints p.i. (F) Vero cell preparations were left untreated or exposed to a mixture of RNAseA and RNAseV-I prior to addition to DC cultures as described in materials and methods. Expression of PKR and IRF7 in DCs was analyzed using qPCR 8 h after addition of Vero cell preparations. (G) DCs were cocultured with Vero cell preparations as described in (D) and protein expression of RIG-I, MDA5 and PKR was analyzed by WB 16 h after start of coculture. (H) DCs were treated as described in (D) and 16 h after start of coculture, cells were harvested, washed and infected with EV9 at an MOI of 1. Data are representative of 3 independent experiments using different donors.

Furthermore, RNAse treatment abrogated the protective effect on EV infection of DCs (**Fig. 4H**), demonstrating the essential role of recognition of viral RNA in the induction of an antiviral state.

Discussion

Phagocytosis of infected cells represents one of the defense mechanisms against viral infection and is executed by different immune cells. Here we report that uptake of CVB3- infected cells by human DCs resulted in rapid increase in both mRNA and

protein levels of viral sensors including the RNA helicases RIG-I and MDA5 and effector molecules like PKR. Upregulation of these ISGs required intact endosomal acidification, was dependent on the presence of viral RNA and could be mimicked by exposure to the synthetic dsRNA analogue poly(I:C). Moreover, DCs that had taken up CVB-infected cells were protected against lethal infection with EV.

Recent mouse studies have highlighted the role of various RIG-like helicases in recognition of viral RNA [15, 17, 22]. These findings have challenged our view of the relative importance of TLRs and non-TLRs in anti-viral immunity. For instance, recognition of the synthetic dsRNA mimetic poly(I:C) was classically thought to be mediated via TLR3 [7], while more recent data implicate MDA5 to be a crucial component for type I IFN production upon poly(I:C) stimulation or viral infection in mice [15, 17]. Very limited data is available regarding these novel RNA sensors in the human setting, but one study shows that poly(I:C) responses in human cell lines are RIG-I dependent [35], suggesting the existence of species-specific or cell-type specific differences, analogues to the species-specific difference in recognition of ssRNAs by TLR7 and TLR8 [9].

We previously demonstrated the dramatic consequences of EV infection on the function and viability of human DCs [25]. We presently document that distinct TLR ligands displayed a remarkably different effect on the induction of viral resistance in DCs. While poly(I:C) rapidly increased the level of different ISGs, including RIG-I and MDA5, and protected DCs against infection, other ligands such as PAM, LTA and R848 did not. LPS caused a modest increase in ISG expression. This might imply that ISG upregulation is mediated in a TRIF dependent fashion, as both poly(I:C) and LPS have been reported to signal via this adaptor protein [36].

To study the effects of viral RNA recognition under conditions that more closely resemble the physiological situation, we used a model system in which DCs acquired virus-infected cells via phagocytosis. Uptake of CVB3-infected cells resulted in ISG upregulation and protection against EV, both of which could be blocked by CQ. As CQ is known to increase endosomal pH and it has been shown that the interaction between poly(I:C)/TLR3 [34] and CpG/TLR9 [37] is pH dependent, this would suggest a role for TLR3 in our experiments. Alternatively, CQ could hamper transport of viral structures, such as dsRNA, to the cytoplasm where recognition by other PPRs like RIG-I or MDA5 could take place. However, a recent study has shown that CQ treatment of human DCs rather increases export of soluble antigen from early endosomes into the cytosol thereby promoting antigen cross-presentation [38]. Identification of the exact routing of viral PAMPs following uptake by DCs can possibly aid in dissecting the

relative contribution of TLRs and RNA helicases to different facets of the antiviral immune response.

It has been shown that, besides viral nucleic acids, viral proteins can also alter the activation status of DCs [39, 40], which could affect DC susceptibility following phagocytosis of infected cells. Our experiments identified RNA within infected Vero cell preparations to be the crucial component for both ISG upregulation and resistance against infection. Since uptake of non-infected cells had no effect, we favor a role for viral RNA in these processes. However, we can not exclude that infection leads to modification of host RNA structures that could be subsequently recognized by different RNA sensors. The use of Vero cells in our experiments showed that phagocytosis of infected cells by DCs can induce protection independently of type I IFN released by infected cells. Thus, recognition of RNA could preserve the ability of DCs to engage an antiviral state, even when type I IFN responses in the infected cells are blocked as a consequence of viral immune evasion strategies.

In conclusion, recognition of viral RNA rapidly induces an antiviral state in human DCs. This might reveal a mechanism by which DCs protect themselves against viruses when attracted to an environment with ongoing infection, thereby facilitating adequate instruction of virus-specific T cells to clear viral infection.

Acknowledgments

The authors would like to thank Bianca Blom (Amsterdam Medical Center/University of Amsterdam, Amsterdam, The Netherlands) for help with the type I IFN blocking experiments. This work was supported by grants from the Netherlands Diabetes Foundation (DFN 2001.00.047) to M.K. and The Netherlands Organization for Scientific Research (NWO-VIDI-917.46.305) to F.J.M.v.K. and (NWO-912-02-034) to G.J.A. P.M.B and P.B.F. were supported by a National Institutes of Health, General Medical Sciences grant (1R01 GM068448).

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Chapter 8

Summary

&

General Discussion

Summary

DCs are master regulators of immunity. They can induce protective immune responses against harmful pathogens, but also contribute to the maintenance of tolerance [1]. For example, DCs are considered to be the most efficient APCs capable of activating naive CD4+ T-cells as well as CD8+ T-cells, crucial for immunity against viral infection or tumor cells. At the same time, DCs can prevent responses to self-Ag by mediating T-cell deletion, anergy or induction of regulatory T-cells (Tregs) [2, 3]. DCs exist in different activation states that have classically been designated as 'immature' (non-activated) or 'mature' (activated). In this view, immature DCs are tolerogenic, while mature DCs have the capacity to prime effector T-cell responses. However, the term mature is commonly used as a phenotypic rather than a functional description for DCs expressing high cell-surface levels of MHC and costimulatory molecules like CD80 and CD86. In recent years it has become apparent that phenotypically mature DCs do not always promote T-cell immunity and can, in fact, induce tolerance [4, 5]. Many signals a DC receives from its environment can induce at least some aspects of DC maturation. However, it is now appreciated that especially activation of pattern recognition receptors (PRRs) drives the transformation of immature DCs into mature DCs displaying full effector function [6, 7]. Thus, although DCs activated by for instance inflammatory mediators alone can support T-cell proliferation, they are poor inducers of effector T-cells. In contrast, DCs stimulated via different PRRs have the capacity to generate Th1, Th2, Th17 and possibly even Treg responses [8-11], a feature that might be exploited by several microorganisms or even tumor cells to escape the immune system. Cross-talk between different PRRs can further fine tune the DC activation program and thus the immune response that is being initiated.

Not surprisingly, considering the important function of PRRs in induction of immune responses, these receptors also play a role in several inflammatory disorders. The research described in this thesis has focused on several aspects of PRR biology in health and disease, particularly the effects on DC function. **Chapter 2** gives an overview of our current understanding of the contribution of two important PRR families; the TLRs and NLRs, in the pathogenesis of several autoimmune and auto-inflammatory disorders. In **chapter 3** we investigated the expression levels of TLRs, NLRs, CLRs and RLHs in human monocyte-derived DCs under pro- and anti-inflammatory conditions. In addition, detailed analysis of PRRs specialized in sensing RNA demonstrated that human plasmacytoid DCs express significantly higher levels of TLR7, RIG-I, MDA5 and PKR compared to myeloid DC subsets, indicating that pDCs have evolved to function in an environment with high virus burden. Finally, we showed that

cross-talk between TLR-matured pDCs and moDC can induce a state of viral resistance in moDCs characterized by enhanced RLH expression and protection against picornavirus infection. In **chapter 4** the effect of the Crohn's disease (CD) associated NOD2 3020insC mutation (NOD2fs) on DC function was analyzed. Although studies in mice have suggested that NOD2fs could represent either a loss-of function or a gain-of-function mutation, we showed here that this mutation results in a loss-of-function phenotype in human moDCs. While NOD2fs moDCs responded normally to purified TLR ligands, they failed to upregulate costimulatory molecules in response to the NOD2 ligand muramyldipeptide (MDP). Moreover, these cells did not display the MDP-induced enhancement of TLR-mediated production of pro- and anti-inflammatory cytokines that was observed in control moDCs. These findings imply that release of imbalanced cytokine profiles by DCs could contribute to NOD2fs associated CD. **Chapter 5** describes the role of NOD2 in synthesis and release of IL-1β, one of the most potent pro-inflammatory cytokines. In this chapter we used human peripheral blood mononuclear cells (PBMCs), since these cells are far better producers of IL-1β compared to moDCs. By investigating the responses of PBMCs from CD patients homozygous for the NOD2fs mutation, we showed that NOD2 is involved in (I) proIL-1β mRNA transcription (II) release of bioactive IL-1β and (III) synergy between NOD2 and TLRs for IL-1β production, indicating that NOD2 is essential for normal IL-1β responses in human PBMCs.

In the next part of the thesis we focused on the interaction between human moDCs and viruses belonging to the human enterovirus-B (HEV-B) species. HEV-Bs are common worldwide and clinical outcome following infection is generally mild. However, these viruses have a long-standing association with disorders like type 1 diabetes mellitus and myocarditis and it has been suggested that they may trigger an autoimmune process. **Chapter 6** describes the effect of exposure of moDCs to different coxsackie B viruses (CVB) and echoviruses (EV), closely related HEV-B serotypes. We showed that moDCs are susceptible to EV, but not CVB infection, *in vitro*. Interestingly, EV infection did not induce moDC activation, but rapidly impeded TLR-mediated responses and DC viability. In order to explain the apparent discrepancy between the dramatic effects of EV infection on moDCs *in vitro* and the usually mild clinical outcome of HEV-B infections, we attempted to identify factors that could alter susceptibility of DCs for infection. This is discussed in **chapter 7** where it is shown that phagocytosis of CVB-infected cells can induce an antiviral state in human moDCs. Uptake of infected cells increased the expression of the cytoplasmic RLHs RIG-I and MDA5 as well as anti-viral effector molecules like PKR and protected moDCs against subsequent EV9 infection. These effects depended on recognition of viral RNA and an

acidic pH within the endosomal compartment, suggesting a role for TLRs. The induction of an antiviral state following recognition of viral RNA provides a possible mechanism by which DCs can protect themselves against viruses when attracted to an environment with ongoing infection.

General Discussion

The NOD2 3020insC mutation

NOD2 (CARD15) is a cytoplasmic PRR that can be activated by the muramyldipeptide (MDP) moiety of the bacterial cell wall component peptidoglycan (PGN), leading to activation of NFκB and cytokine production [12]. Although MDP stimulation in human DCs on itself results in relative modest cytokine induction [9, 13], cross-talk between TLR and NOD2 pathways leads to synergistic release of both pro- and anti-inflammatory cytokines, an effect that has been observed in different cell types [9, 14, 15]. NOD2 has received a great deal of attention in recent years, because of the association between mutations in this NLR family member and susceptibility to Crohn's disease (CD) that was discovered in 2001 [16, 17]. Three main NOD2 variants exhibit the strongest association with CD, in particular the frameshift mutation 3020insC (NOD2fs). The NOD2fs mutation alters the C-terminal leucine-rich-repeat (LRR) region of the protein, thought to be involved in ligand sensing, and cell lines transfected with NOD2fs show defective NFκB signaling [16]. Despite the strong association with CD (individuals homozygous for the NOD2fs allele show an up to 40-fold increased susceptibility), mutations in NOD2 are neither sufficient nor required for the development of the disease and it has been shown that even homozygous NOD2fs carriers do not necessarily exhibit gastrointestinal complaints [18]. This finding suggests that additional genetic and/or environmental factors are required for intestinal inflammation to occur and underscore the fact that CD, like many (autoimmune) diseases, is a true multi-factorial disorder.

NOD2 mutation and Crohn's disease: animal models and human data

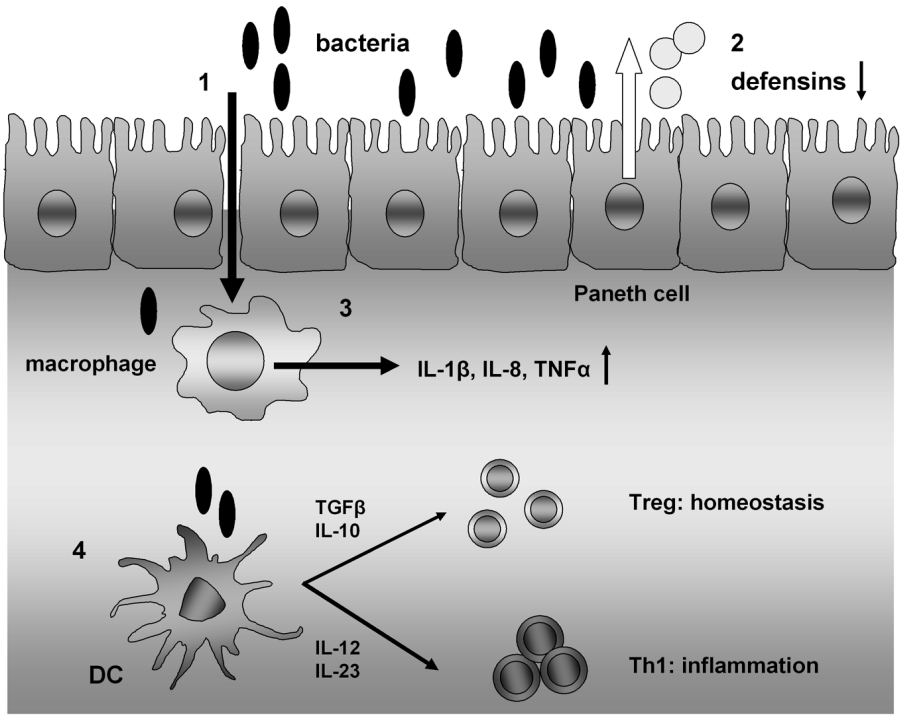
There has been a lot of debate regarding the mechanism by which mutations in NOD2 could contribute to the pathogenesis of CD. A number of mouse studies performed in the Strober lab have suggested that NOD2 might function as a negative regulator of TLR signaling [19, 20]. In these studies, MDP decreased the TLR-induced production of IL-12 and other pro-inflammatory cytokines by DCs derived from NOD2 wt mice, whereas this effect was not observed with DCs from $NOD2^{-/-}$ mice. In addition, $NOD2^{-/-}$ mice exhibited an increased systemic IL-12 response following administration of PGN

[20]. This suggests that the NOD2fs mutation would result in increased Th1 responses, which is indeed one of the hallmarks of CD [21, 22]. At odds with these results, we showed in **chapter 4** that human moDCs from CD patients homozygous for the NOD2fs mutation produced less IL-12 (among other cytokines) following combined TLR/NOD2 stimulation, while the opposite was the case when using DCs from healthy controls. Others have reported similar results in different human cell types [14, 23]. The reason for this apparent discrepancy might be related to potential distinct effects of the NOD2fs polymorphism compared to that of complete NOD2 deficiency in $NOD2^{-/-}$ mice. This is underscored by the finding that mutations in distinct NOD2 domains are associated with different diseases. For example, while NOD2 mutations in the LRR domain are associated with CD, in Blau Syndrome, an inflammatory disorder characterized by inflammation of the joints, eyes and skin [24], mutations affect the NOD domain. It has been hypothesized that the NOD and LRR domains of NOD2 self-associate when the protein is in a 'resting state' to prevent spontaneous activation. The mutations in the NOD domain that cause Blau syndrome could interfere with this auto-inhibitory loop, resulting in a mutated NOD2 protein that is overly sensitive to activation or is even constitutively active [25, 26]. Whether these mechanisms could also play a role in NOD2fs associated CD is currently unknown. Another difference between our study and the work published by the Strober lab is that, unlike what is observed using mouse DCs, TLR2 stimulation of human DCs elicits only weak (if any) IL-12 production, but rather favors a Th2 response [27-29]. This is also shown in **chapter 4** where we demonstrated that human moDCs (in the absence of additional stimulation with IFNγ) do not produce IL-12 upon exposure to Pam_3Cys, a synthetic TLR2 ligand. This suggests that species-specific differences in TLR/NLR biology in humans and mice could explain some of the divergent results.

The association between the observed decrease in cytokine production as a result of NOD2fs and CD might seem paradoxical at first sight, but can possibly be explained when taking into account the special properties of gut residing DC populations, that are considered 'commanders in chief' for mucosal immunity [30]. Different subtypes of intestinal DCs are present in various compartments in the gut, such as the Peyer's patches [31], lamina propria [32] or mucosa-associated lymphoid tissue (MALT) [33]. The responses induced by gut DCs, which are continuously exposed to Ag and microorganisms, are carefully adjusted in order to prevent excessive inflammation. It has been demonstrated that DCs within Peyer's patches or the lamina propria of the healthy gut have the tendency to produce immunosuppressive cytokines like IL-10 rather than inflammatory Th1 promoting cytokines such as IL-12 [34, 35]. In addition, several gut DCs seem to contribute to maintenance of tolerance by expanding the

repertoire of Tregs via a process dependent on TGFβ [36, 37]. As we have shown that not only production of pro-inflammatory cytokines, but also production of IL-10 is defective in NOD2fs DCs, the NOD2fs polymorphism could potentially disturb the delicate balance between pro- and anti-inflammatory cytokines in these specialized DC subsets, leading to awry T-cell responses. Although challenging, experiments using freshly isolated intestinal DC populations, for instance obtained from resection material, could test the validity of this hypothesis.

Figure 1



Possible mechanisms by which NOD2fs can contribute to the pathogenesis of CD. (1) Increased permeability of the intestinal epithelial barrier, leading to enhanced penetration of commensal (and pathogenic) bacteria through the intestinal wall which could contribute to abnormal inflammatory responses. (2) Decreased Paneth cell mediated production of defensins, resulting in impaired mucosal antibacterial activity. (3) Increased production of proinflammatory cytokines (*e.g*. by intestinal macrophages) due to loss of NOD2/TLR cross-tolerance normally mediated via chronic NOD2 stimulation by MDP from intestinal microorganisms. (4) Derailed cytokine production by intestinal DCs. In the normal situation, DCs in the gut have a tendency to produce high levels of anti-inflammatory cytokines (e.g. IL-10, TGFβ) in stead of pro-inflammatory cytokines (*e.g*. IL-12) in order to prevent unwanted inflammatory responses towards commensal bacteria. NOD2fs could disturb this delicate cytokine balance and induce aberrant T-cell responses.

Interestingly, two recent studies might offer another explanation for the association between the NODfs and CD. As mentioned above, 'short term' exposure of human immune cells to MDP in combination with TLR ligands induces release of cytokines in a synergistic fashion. In contrast, independent studies done by Kullberg and Hedl show that 'long term' stimulation of PBMC or monocyte-derived macrophages with MDP can decrease responsiveness towards particular bacterial products and intestinal microorganisms and increases expression of the regulatory molecule IRAK-M [38]. These tolerizing phenomena induced by long term stimulation of NOD2 were lost in cells derived from homozygous NOD2fs individuals, leading to uninhibited release of TNFα and other pro-inflammatory cytokines [38, 39], which may provide additional insight into how NOD2fs could contribute to the pathogenesis of CD. Finally, it should be realized that mutations in NOD2 can influence other aspects of (intestinal) immunity, like deregulated Th17 induction [9], increased intestinal permeability [40, 41] and decreased production of defensins or other anti-microbial proteins/peptides which could affect the mucosal anti-bacterial barrier [42]. It is likely that the effects of NOD2fs can collectively culminate in the deleterious inflammatory responses characteristic of CD (**Fig. 1**).

Restoring the balance in Crohn's disease

Since many years, the mainstay of CD treatment has been the use of anti-inflammatory and immune modulating drugs such as 5-aminosalicylate derivates, corticosteroids and azathioprine. Despite the availability of this arsenal of drugs, surgery is still frequently needed in refractory patients or to treat complications, such as stenosis or perforations [43]. The ultimate goal of CD therapy is local restoration of immune homeostasis in the gut which could replace the need for systemic drug administration that holds a high risk for adverse side-effects [44, 45]. The beneficial effects of neutralizing antibodies against IL-12p40 [46, 47] and TNFα [48, 49] reflect the contribution of deregulated cytokine production in the disease process. Besides neutralization of pro-inflammatory cytokines, the use of immunosuppressive cytokines like IL-10 could be a valuable approach. It has been shown that IL-10 can be effective in reducing intestinal inflammation in several mouse models [50, 51]. Although administration of recombinant human IL-10 has proven to be a relatively safe and well-tolerated treatment that can induce clinical improvement [52-54], it is currently not used for the treatment of CD. The efficacy of therapy with immunosuppressive cytokines could theoretically be even further improved if local administration in the gut could be realized. One strategy that is currently being tested in clinical trials is the

use of genetically modified bacteria that can locally produce anti-inflammatory mediators, which has shown promising results in animal studies [55-57]. Preliminary trials in CD patients with genetically modified *Lactococcus lactis* producing human IL-10 [58] might provide hope for the future.

NOD2 and IL-1β

IL-1β is one of the most potent soluble mediators of inflammation and minimal levels can induce fever, enhanced expression of adhesion molecules in leucocytes and production of other pro-inflammatory cytokines such as IL-6. Considering its powerful pro-inflammatory effects, production of IL-1β is tightly regulated in three consecutive phases. First, signals leading to activation of the transcription factor NFκB can induce mRNA transcription of pro-IL-1β, which is subsequently translated into pro-IL-1β protein. Second, the pro-IL-1β precursor protein needs to be cleaved by caspase-1 (also known as *interleukin-1 converting enzyme*; ICE) into 'mature' IL-1β. Finally, secretion of mature IL-1β into the extracellular space takes place, which is thought to occur independently of its intracellular production [59, 60].

A great deal of controversy currently surrounds the processes involved in regulation of IL-1β production. This controversy has especially gained momentum since the identification of caspase-1 activating multiprotein platforms that were christened *inflammasomes* by the Tschopp group [61]. Key components of inflammasomes are NALP family members (especially NALP3 and NALP1 have been extensively studied) and the adapter protein ASC [62]. Activation of inflammasomes has been reported to result in recruitment and activation of inflammatory caspases that can process pro-IL-1β into 'mature' IL-1β. A large number of papers have claimed that very diverse stimuli can activate the inflammasome, including bacterial and viral RNA, endogenous structures such as uric acid or ATP and, surprisingly, the NOD2 ligand MDP [63-67]. Moreover, it has been put forward that NALPs are indispensable for processing of IL-1β and that 'classical' inflammatory stimuli such as LPS would induce IL-1β only as a result of contamination with NALP stimulating ligands [67]. In the model proposed by Tschopp and colleagues, TLR ligands like LPS are capable of inducing pro-IL-1β via the activation of NFκB, but inflammasome activation is required for subsequent caspase-1 activation and IL-1β processing. Although this model seems to be supported by data obtained in cell lines and in mouse macrophages, it clearly contradicts many earlier studies showing abundant production of IL-1β by monocytes following stimulation with LPS or lipopeptides (some of which synthetic, thus limiting possibility of contamination with NALP activation structures).

Unpublished work done by Netea and colleagues might provide more insight into the complex mechanisms of IL-1β production in monocytes and other cell types in the human setting. They showed that human monocytes and macrophages display strikingly different requirements for release of bioactive IL-1β. While monocytes excreted IL-1β after stimulation with TLR ligands (like LPS) alone, macrophages required two 'danger signals': a TLR ligand that induces pro-IL-1β, and a danger signal such as ATP that can both activate the NALP3 inflammasome and result in the poorly understood K^+-efflux dependent secretion of IL-1β [60, 66]. These differences were shown to be related to the presence of constitutively activated caspase-1 in monocytes, which is in its turn dependent on the inflammasome components ASC and NALP3 (personal communication Mihai Netea and Gerben Ferwerda). This 'one-step' versus 'two-step' principle of IL-1β production in monocytes and macrophages, respectively, might represent adaptation of these cells to their respective environments. While monocytes need to react promptly to microbial structures present in (otherwise sterile) blood, macrophages, which are often located in an environment with frequent exposure to microorganisms (*e.g.* the lung and intestinal mucosa), do not. A potent IL-1β response at each encounter of macrophages with PAMPs might even induce potentially harmful tissue inflammation. Thus, although inflammasome constituents are considered to be crucial components in the IL-1β machinery, activation of NALPs via microbial or endogenous danger signals is not an absolute prerequisite for the production of this pro-inflammatory cytokine.

Taking into account the finding that NALP activation is expandable for induction of IL-1β production and since it has been shown that MDP can induce the release of IL-1β, we investigated the potential role of the MDP sensor NOD2 in IL-1β production in **chapter 5**. Hereto, we extended our experiments on NOD2 function from DCs to PBMCs, since these latter cells (most likely the monocyte-component of PBMCs) are far more efficient producers of this cytokine. The results we obtained by using cells derived from NOD2fs CD patients showed that functional NOD2 is essential for (I) MDP induced IL-1β mRNA transcription and release of bioactive IL-1β and (II) synergy for IL-1β production following combined stimulation with TLR ligands and MDP. Thus, our data show that at least NOD2 is required for a normal MDP-induced IL-1β response in human PBMCs. It is currently not known whether NOD2 and NALPs interact in the processes leading to IL-1β production.

Interaction between DCs and enteroviruses

Enteroviruses and disease

The species Human enterovirus-B (HEV-Bs) within the Enterovirus genus of the family of *Picornaviridae* (picornaviruses) consists of over fifty serotypes, among which several closely related coxsackieviruses (CVBs) and echoviruses (EVs). HEV-Bs are present in water, soil, vegetables and other food items and are most likely transmitted via the faeco-oral or respiratory route. Infections are common world wide and outcome is usually mild or even asymptomatic. Nevertheless, systemic spread of HEV-Bs can affect target organs like the heart, brain or pancreas which causes more serious pathology. In fact, infections with HEV-Bs are the leading cause of myocarditis [68] and aseptic meningitis [69]. In addition, HEV-Bs have a long-standing association with type I diabetes mellitus (T1DM) [70]. T1DM is a prototypical auto-immune disease characterized by progressive loss of insulin-producing β-cells in the pancreas due to the actions of auto-aggressive T-cells. Analogous to CD, T1DM is thought to arise due to exposure to certain environmental factors in genetically susceptible individuals. In case of T1DM, CVB- and EV-infections are considered to be important environmental risk factors [71-73]. Many different mechanisms linking HEV-B infection to T1DM have been proposed, such as a direct lytic effect of the virus on pancreatic β-cells [74-76], molecular mimicry between viral and pancreas antigens [77] and bystander activation [78], which do not have to be mutually exclusive. However, both hard proof and consensus are still lacking [79].

Possible links between T1DM and enterovirus-infection of DCs

Little data is present on interaction between T1DM-associated HEV-Bs and human DCs. This is surprising, considering the crucial role of DCs in the decision process between tolerance and immunity and the potential effects of exposure to viruses or viral structures on the activation status of DCs. In **chapter 6**, we investigated whether exposure to CVB or EV strains affected human moDC function. A striking difference was observed in susceptibility of moDCs for infection with different HEV-Bs. While the CVB strains tested did not infect or activate moDCs, various EV strains were capable of establishing a productive infection, which resulted in inhibition of TLR-mediated DC activation and induction of cell death. At first sight, it may be difficult to envision that decreased DC activation and viability upon HEV-B infection would be linked to induction of an autoimmune process. However, defective DC function and subsequently impaired induction of anti-HEV-B T-cell responses could hamper effective eradication of the virus. It has already been shown that HEV-Bs are detected more

frequently in the blood and intestines of diabetic subjects compared to healthy individuals [80-84], suggesting that HEV-B infections are more common or, possibly even more important, have a more persistent character, in T1DM patients. When HEV-Bs infect the pancreas, release of normally sequestered auto-antigens and subsequent activation of autoreactive T-cells [78], as well as direct effects of HEV-B infection on β-cell function or viability [74, 85], could increase the risk of T1DM to develop. Interestingly, autoreactive T-cells with effector function as well as autoreactive T-cells with suppressor functions have been identified in the human setting [86-88] and it has been shown that these T cells display a proinflammatory phenotype in T1DM patients, while in healthy controls they predominantly show a regulatory phenotype [89]. Further investigation is needed to obtain more insight into the potential involvement of DCs in directing autoreactive T-cell differentiation. Data from mouse models have already pointed towards a role for DCs in these processes, since presentation of tissue antigens by 'steady-state' DCs, that reside in the pancreas under non-inflammatory conditions, results in tolerance towards self-Ags [90-92]. Furthermore, 'tolerogenic' DCs possess the capacity to increase the number and function of Treg exerting islet-specific immune suppression, which can prevent autoimmunity or even revert the disease process in animals that have developed overt diabetes [93-95]. These findings might open new perspectives for cell-based therapy of autoimmune diseases like T1DM.

Protection of DCs against viral infection

Although we showed that EV can dramatically impair DC function *in vitro*, these viruses are not commonly associated with severe immune pathology, which would likely occur if massive decimation of DCs by EV infection would take place. Therefore, we hypothesized that DCs attracted to an infected area *in vivo* must somehow be protected against viral infection. In **chapter 7** we showed that moDCs accrued an antiviral state characterized by the upregulation of cytoplasmic viral sensors and antiviral effector molecules following exposure to the synthetic dsRNA analogue poly(I:C). Importantly, protection from viral infection was also induced following phagocytosis of dead CVB-infected cells, a model that more closely resembles the physiological context in which DCs encounter viral RNA. Our study further revealed that induction of the antiviral state in DCs was mediated via (viral) RNA in infected cells, but not type I IFN production by the infected cells, since we used Vero cells that have a genetic defect in type I IFN synthesis. The RNA-mediated protection against infection might represent an important 'safety backup' for DCs present in infected

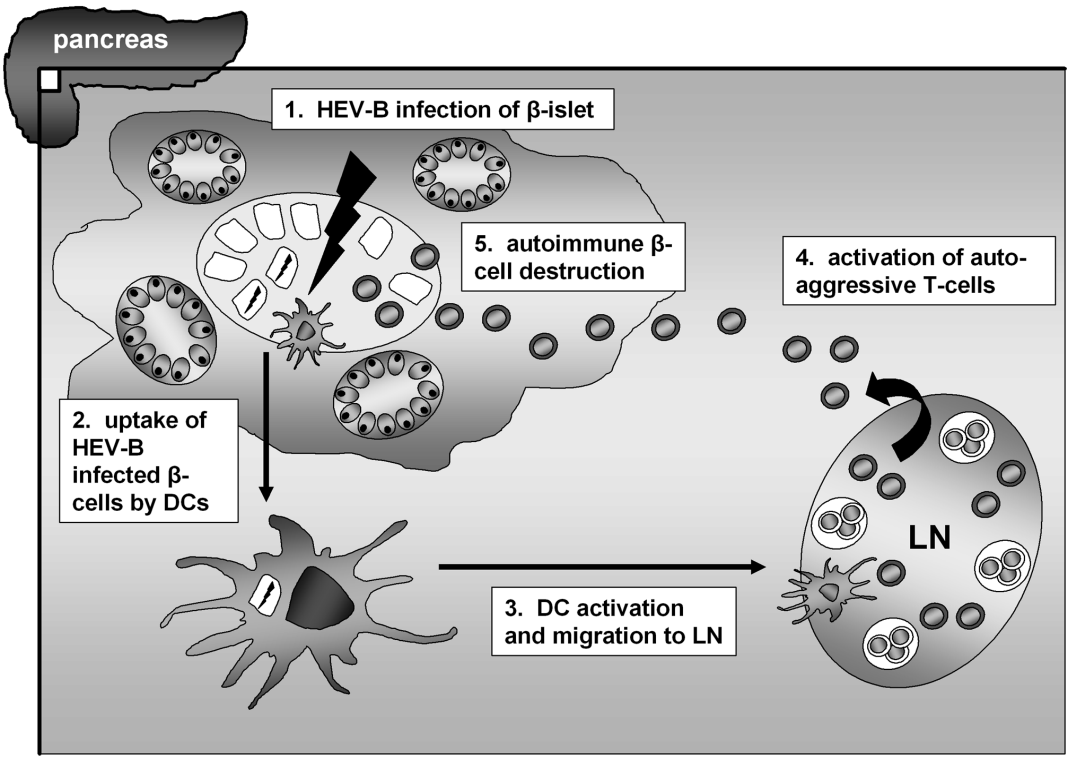
tissues, since many viruses have the capacity to block type I IFN responses [96, 97] that could normally trigger an antiviral program in neighboring cells.

At present, there is lack of consensus concerning the exact role of TLRs and RLHs in the response against viral RNA. This is possibly related to the use of different read outs, reagents and cell types. Importantly, most data regarding RLHs is derived from mouse studies, while the role of these molecules in the response to viruses in the human setting is virtually unknown. Our experiments did not pinpoint which RNA sensor in DCs mediated the observed protection against infection following exposure to viral RNA. A likely candidate would be MDA5, since this RLH is reportedly involved in the recognition of picornaviruses and poly(I:C) in mouse bone marrow-derived DCs [98, 99]. However, the results described in **chapter 7** show that both upregulation of cytoplasmic viral sensors and resistance against EV infection following exposure to poly(I:C) or phagocytosis of virus-infected cells were dramatically impaired following pretreatment of moDCs with chloroquine (CQ), a chemical that raises endosomal pH. Since interaction between poly(I:C) and TLR3 is dependent on an acidic pH [100], this would rather suggest a role for TLR3 in induction of an antiviral state in human DCs. Alternatively, a model could be envisioned in which both TLRs and MDA5 are involved. In such a model, activation of TLRs following exposure of DCs to poly(I:C) or uptake of infected cells might directly or indirectly (via production of type I IFN) enhance the expression of RLHs. Increased levels of MDA5 could subsequently ensure a rapid and robust antiviral response following recognition of EV9 RNA in the cytoplasm and prevent a lethal infection of the DC.

Phagocytosis of infected cells and DC activation

Activation of DCs upon uptake of infected islet cells could provide an explanation for the association between HEV-B and T1DM. As discussed above, systemic HEV-B infections can cause infection of pancreatic β-cells, which directly affects their insulin secreting capacity or viability [85, 101, 102]. In case of a lytic infection of β-cells with HEV-B, recognition of viral RNA following phagocytosis of dead β-cells by DCs could (I) prevent DCs from becoming productively infected with HEV-B themselves and (II) induce DC activation and presentation of islet-antigens in the genetically susceptible host. In this respect, it is interesting to note that genetic susceptibility seems to be related to particular HLA genotypes. Both protective as well as susceptible HLA alleles have been identified [103].

Figure 2



Model of the potential intermediary role of DCs in the association between HEV-B and T1DM. Following HEV-B infection of pancreatic islets (1), dead β-cells are taken up by tissue resident DCs (2). Danger signals as a result of viral infection (*e.g.* dsRNA, pro-inflammatory cytokines) induce activation and migration of DCs to the draining lymph nodes (LN) (3), where activation of effector T-cells specific for pancreatic antigens can occur in the susceptible host. These auto-aggressive T-cells can cause destruction of β-cells and induce the autoimmune process characteristic of type I diabetes (5).

Presentation of particular pancreatic peptides by DCs in the context of viral infection (*i.e.* 'danger') could result in autoreactive effector T-cell responses that mediate further β-cell damage and consequently boost the autoimmune process (**Fig. 2**).

Surprisingly, although we showed that phagocytosis of HEV-B-infected cells induced upregulation of RLHs and protection against EV infection, it did not result in overt phenotypic DC maturation or production of the proinflammatory cytokines TNFα or IL-12, in contrast to what was observed following stimulation of DCs with poly(I:C) (data not shown). Possibly, additional signals next to viral RNA in the phagocytosed cargo could be required for full DC activation to occur, which might be a safety mechanism to avoid unwanted responses following phagocytosis of apoptotic cell material that can

contain many different host RNA species. Potential factors that could contribute to enhanced maturation and cytokine production by DCs include exposure to pro-inflammatory cytokines or ligation of CD40 [104-106], for instance following interaction with T-cells in the secondary lymphoid organs. It will be interesting to determine the nature of DC activation following uptake of islet cells in absence or presence of such signals *in vitro.* Experiments in which DCs that have taken up uninfected or infected islet cells are allowed to interact with undifferentiated T-cells specific for β-cell antigens could demonstrate whether DCs might form a missing link between HEV-B infections and the auto-aggressive T-cell responses characteristic of T1DM.

Future directions

Extensive research over the past decade has demonstrated the crucial involvement of PRRs in both adaptive and innate immune responses. For example, input via PRRs helps DCs to determine the type of T-cell response that needs to be generated under particular conditions [107, 108], whereas PRR triggering on neutrophils can mediate release of cytokines, chemokines and reactive oxygen intermediates [109]. The vital importance of these receptors in defense against pathogens becomes especially apparent in individuals with defective PRR function. For instance, mutations in IRAK-4, a signaling molecule downstream of many TLRs, result in increased susceptibility for severe pneumococcal and staphylococcal infections [110], while individuals with polymorphisms in the dsRNA sensor TLR3 are prone to herpes simplex encephalitis [111]. It could be valuable with regards to a rational design of therapeutic strategies to have extensive knowledge regarding the exact contribution of PRRs to immune regulation in the human setting. For instance, use of particular PRR agonists as adjuvants in vaccinations against infectious diseases, or even cancer, could enhance CD4+ and CD8+ T-cell responses and help to overcome the generally weak response to tumor-antigens, that may display few differences from normal self-antigens. However, a significant proportion of the currently available data involving PRR biology and immunity to infection is derived from animal studies. Although especially mouse models are a useful tool for studying involvement of particular cell types or molecules in the modus operandi of the immune system, we should be well aware that significant differences between the immune systems of humans and mice exist [112] that can potentially prevent a successful therapy in mice from becoming a successful therapy in humans. Therefore, it might be rewarding to put more emphasis on verifying and

extending findings done in animal models in humans. In order to achieve maximal results, a tight collaboration between research institutes and hospitals as well as increased efforts to develop research tools and techniques that can be used in the human setting will be required.

Besides the function of PRRs in induction of immunity against microorganisms, unregulated PRR responses have been implicated in inflammatory disorders such as Crohn's disease or rheumatoid arthritis. Here, too, a detailed insight in the mechanisms linking PRRs to disease pathogenesis can be helpful in designing a therapeutic intervention. One illustrative example is the finding that mutations in the NLR family member NALP3 are associated with a number of rare auto-inflammatory disorders like *Muckle-Wells syndrome* and *familial cold autoinflammatory syndrome* [113-115]. More in-depth analysis subsequently demonstrated that the mutations in NALP3 result in uncontrolled release of the pro-inflammatory cytokine IL-1β [116] and the significance of this finding has been substantiated by the excellent response of these auto-inflammatory diseases to treatment with IL-1 receptor antagonists [117-120]. Given the fact that NLRs and other PRRs control production of these inflammatory mediators, targeting the initial PRR pathway involved is possibly an even more effective approach. In fact, the value of synthetic TLR antagonists for the treatment of immune mediated disorders like severe sepsis and SLE is currently being tested in clinical trials [121]. Perhaps paradoxically, investigation of the immune evasion tactics displayed by pathogens, especially viruses, might help us to better understand how to manipulate PRR pathways for our own benefit. The evolutionary determined optimization of viral proteins is expected to have led to maximal inhibition of a particular host immune function and can be regarded as a 'natural drug development program'. Since viruses often strategically target crucial host signaling proteins, studying host-pathogen interaction can potentially point the way to key drug targets and open up novel therapeutic avenues for the treatment of inflammatory disorders.

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Nederlandse samenvatting

Samenvatting

Het immuunsysteem van de mens bestaat uit twee aparte, maar nauw verwante onderdelen: het aangeboren (innate) en het verworven (acquired) immuunsysteem. Het aangeboren immuunsysteem bestaat uit o.a. granulocyten, macrofagen en monocyten. Wanneer deze cellen micro-organismen zoals bacteriën signaleren, zijn ze in staat direct te reageren door middel van excretie van pro-inflammatoire cytokines, antimicrobiële eiwitten en reactieve zuurstofmetabolieten. Het verworven immuunsyteem wordt gevormd door T- en B-lymfocyten. De belangrijkste functie van B-lymfocyten is productie van antilichamen die kunnen binden aan micro-organismen waardoor deze makkelijker herkend en opgeruimd kunnen worden. T-cellen zijn er in verschillende soorten: helper T-cellen (Th), cytotoxische T-cellen (CTL) en regulatoire T-cellen (Treg). De diverse Th subtypes hebben een belangrijke ondersteunende taak en zorgen voor een optimale functie van CTLs, B-cellen en de cellen van het aangeboren immuunsysteem. CTLs zijn in staat zijn om geïnfecteerde lichaamscellen of tumorcellen te herkennen en deze direct te doden, zodat de infectie of tumorgroei geremd wordt. Soms wordt echter door cellen van het verworven immuunsysteem ook een lichaamseigen structuur herkend. Dit kan leiden tot een afweerreactie tegen deze structuren en het ontstaan van auto-immuunziekten, zoals diabetes of reumatoïde artritis. Treg spelen een belangrijke rol bij het voorkomen van zulke reacties tegen lichaamseigen structuren en het dempen van heftige ontstekingsreacties om weefselschade te voorkomen. Ze werken o.a. via het remmen van de deling en functie van effector T-cellen.

Een belangrijk verschil tussen cellen van het aangeboren en verworven immuunsysteem is, dat de cellen van het verworven immuunsysteem heel specifiek reageren op één bepaald molecuul, een antigeen genoemd. Antigenen kunnen delen van een micro-organisme zijn, of van andere lichaamsvreemde (en soms dus lichaamseigen) structuren. Het antigeen wordt herkend via een specifieke receptor op het celoppervlak. Een receptor bindt één antigeen, zoals een sleutel doorgaans maar in één slot past. Herhaalde blootstelling van de cel aan hetzelfde antigeen resulteert in een snellere en verbeterde afweerrespons. Dit fenomeen heet ‘immunologisch geheugen’ (memory) en is de gedachte achter het gebruik van vaccinaties die immuniteit moeten bieden tegen bijvoorbeeld difterie of kinkhoest. Een ander verschil met de cellen van het aangeboren immuunsysteem is, dat T-cellen hun functie niet kunnen uitoefenen voordat ze hun specifieke antigeen ‘gepresenteerd’ hebben gekregen. Cellen die antigeen presenteren aan T-cellen en deze zodoende kunnen activeren worden *antigeen presenterende cellen* (APC) genoemd. De meeste efficiënte APC is de dendritische cel.

Dendritische cellen (DCs) komen voor in vrijwel alle weefsels en organen, met name op plaatsen waar sprake is van een overgang tussen het interne en externe milieu, zoals de huid, longen en darmen. In deze weefsels is de DC op zoek naar potentieel ziekteverwekkende micro-organismen (pathogenen), zoals bacteriën of virussen. DCs herkennen pathogenen met behulp van speciale receptoren: de zogenaamde Pattern

Recognition Receptors (PRRs). Er bestaan veel verschillende PRRs en iedere receptor is in staat een structuur binnen een klasse van micro-organismen te herkennen, bijvoorbeeld onderdelen van de bacteriële celwand of viraal RNA. Herkenning van een pathogeen via PRRs kan ertoe leiden dat de DC het betreffende organisme opneemt via een proces dat fagocytose wordt genoemd. Het organisme wordt dan binnen de DC verteerd en verschillende stukjes van dit organisme worden vervolgens als antigeen op het celoppervlak van de DC gepresenteerd. Een ander belangrijk gevolg van de herkenning van microbiële structuren door PRRs is, dat de DC een activatieproces ondergaat dat *maturatie* wordt genoemd. Maturatie van een DC is een ingewikkeld fenomeen dat wordt gekenmerkt door het selectief aan- en uitschakelen van zeer veel genen, wat resulteert in een DC met andere uiterlijke kenmerken en andere eigenschappen. Dit maturatie proces is zeer belangrijk voor de uitkomst van de interactie tussen DCs en T-cellen, die plaatsvindt in lymfoïde weefsels zoals de lymfeklieren. Mature (geactiveerde) DCs zijn de meest efficiënte APCs die we kennen en veroorzaken een sterke T-cel activatie en dus een krachtige immuunrespons, bijvoorbeeld in het geval van een virus infectie. Naast T-cel activatie, speelt de DC ook een cruciale rol in de ontwikkeling van verschillende soorten Th-cellen. Deze ontwikkeling wordt voor een belangrijk deel bepaald door middel van boodschapper-eiwitten (cytokines) geproduceerd door de DC. De signalen die een DC ontvangt in (geïnfecteerde) weefsels, met name via PRRs, bepalen welke cytokines hij produceert en dus welk soort Th-cel wordt gevormd. Hierdoor zijn DCs onmisbaar voor de inductie van een immuunrespons die precies is afgestemd op het desbetreffende micro-organisme. Immature (ongeactiveerde) DCs, daarentegen, voorkómen of dempen juist immuunresponsen. Antigeen presentatie door een DC die *niet* is geactiveerd via PRRs (dus in *afwezigheid* van infectie) leidt bijvoorbeeld tot het uitschakelen van T-cellen die zouden kunnen reageren op lichaamseigen structuren of de ontwikkeling van Treg. Op deze manier speelt de DC een belangrijke rol bij de beslissing tussen immuniteit en tolerantie.

Gezien hun belang in de regulatie van immuunresponsen, is het niet verwonderlijk dat een defect in PRR- of DC-functie een rol kan spelen bij het ontstaan van diverse inflammatoire en infectieuze aandoeningen. Het onderzoek beschreven in dit proefschrift richt zich op verschillende aspecten van PRRs tijdens gezondheid en ziekte, met name de effecten op DC functie. **Hoofdstuk 2** geeft een overzicht van de bijdrage van twee belangrijke PRR families: de Toll-like receptors (TLRs) en de NOD-like receptors (NLRs), in de pathogenese van verschillende autoimmuun en auto-inflammatoire aandoeningen. In **hoofdstuk 3** hebben we gekeken naar de expressie van vier belangrijke groepen PRRs: TLRs, NLRs, C-type lectin receptors (CLRs) en RIG-like helicases (RLHs) in humane monocyte-derived DCs (moDCs) onder verschillende omstandigheden. Tevens hebben we in verschillende humane DC subtypes de levels van PRRs die specifiek reageren op viraal RNA in detail bestudeerd. Deze experimenten toonden aan dat plasmacytoïde DCs (pDCs) een hogere expressie hebben van TLR7, RIG-I, MDA5 en PKR dan myeloide DC subsets, wat mogelijk illustreert dat pDCs zich hebben aangepast om te functioneren in een virus-rijke omgeving. Tenslotte hebben we in dit hoofdstuk laten zien dat communicatie tussen TLR-geactiveerde pDCs en

moDCs een antivirale staat in moDCs kan opwekken, die gekarakteriseerd wordt door verhoogde expressie van RLHs en bescherming tegen picornavirus infectie.

In **hoofdstuk 4** is het effect van de met de ziekte van Crohn (CD) geassocieerde mutatie in NOD2 (NOD2 3020insC, NOD2fs) op de functie van humane moDCs bestudeerd. Ondanks een normale respons van NOD2fs DCs op gezuiverde TLR liganden, vertoonden deze cellen geen verhoogde expressie van costimulatoire moleculen (CD80, CD86) na blootstelling aan MDP. Verder resulteerde gecombineerde TLR/NOD2 stimulatie van NOD2fs DCs niet in de typisch synergistisch verhoogde productie van pro- en anti-inflammatoire cytokines, wat wel het geval was bij controle DCs. Deze bevindingen tonen aan dat NOD2fs in humane moDCs leidt tot onvermogen om te reageren op het NOD2 ligand MDP en suggereren dat een verstoorde regulatie van de cytokineproductie in NOD2fs DCs zou kunnen bijdragen aan NOD2fs geassocieerde CD. **Hoofdstuk 5** beschrijft de rol van NOD2 in de synthese en uitscheiding van IL-1β, een van de meest potente pro-inflammatoire cytokines. In dit hoofdstuk maken we een uitstap naar humane perifere bloed mononucleaire cellen (PBMCs), omdat deze cellen meer IL-1β maken in vergelijking met humane DCs. Door de respons van PBMC van CD patiënten met de NOD2fs mutatie te bestuderen, hebben we kunnen aantonen dat NOD2 betrokken is bij: (1) proIL-1β mRNA productie (2) uitscheiding van het actieve IL-1β en (3) synergie tussen NOD2 en TLRs met betrekking tot de productie van IL-1β. Dit impliceert dat NOD2 een essentiële bijdrage levert aan normale IL-1β responsen in humane PBMCs.

In het volgende deel van het proefschrift hebben we ons gericht op de interactie tussen humane moDCs en humane enterovirus B (HEV-B) species. HEV-Bs zijn wereldwijd voorkomende virussen en de klinische gevolgen van infectie zijn veelal mild. Echter, reeds sinds de jaren ’60 worden infecties met HEV-B geassocieerd met type 1 diabetes mellitus (T1DM), zonder dat het onderliggende mechanisme van deze associatie bekend is. Naast T1DM, zijn HEV-B mogelijk ook betrokken bij het ontstaan van myocarditis en de ziekte van Sjögren en het wordt gesuggereerd dat deze virussen een autoimmuunproces kunnen induceren. In **hoofdstuk 6** beschrijven we het effect van blootstelling van humane moDCs aan verschillende coxsackievirussen (CVB) en echovirussen (EV), twee nauw verwante HEV-Bs serotypes. De experimenten beschreven in dit hoofdstuk tonen aan dat moDCs zeer efficiënt geïnfecteerd kunnen worden met verschillende EV, maar niet met CVB serotypes. Infectie resulteerde niet in DC activatie, maar in een verminderd vermogen van DCs om te reageren op TLR liganden. Bovendien induceerde EV infectie celdood binnen 24 uur. Gezien de discrepantie tussen het dramatische effect van *in vitro* infecties en het gewoonlijk milde klinische beeld van infecties *in vivo*, hebben we getracht factoren te identificeren die de gevoeligheid van DCs voor infectie zouden kunnen beïnvloeden. Deze factoren worden besproken in **hoofdstuk 7**, waar we laten zien dat fagocytose van CVB-geïnfecteerde Vero cellen (die een defect in type I IFN synthese hebben) een antivirale staat kan opwekken in humane moDCs. Opname van geïnfecteerde Vero cellen leidde tot een verhoogde expressie van de RLHs RIG-I en MDA5 en PKR (een antiviraal effector molecuul) en beschermde de DC tegen een infectie met EV. Deze

effecten bleken afhankelijk te zijn van herkenning van viraal RNA en het behoud van een zure pH binnen het endosomale compartiment, wat een rol voor TLRs impliceert. Deze bevindingen zouden een mechanisme kunnen weergeven waarop DCs zich beschermen tegen virussen wanneer ze aanwezig zijn in weefsels met een actieve virale infectie.

In **hoofdstuk 8** worden de bevindingen van het onderzoek beschreven in dit proefschrift samengevat en bediscussieerd.

Toekomstperspectief

De afgelopen tien jaar is duidelijk geworden dat PRRs een cruciale rol spelen in de regulatie van zowel aangeboren (innate) als verworven (acquired) immuunresponsen. Deze kennis heeft de zoektocht naar mogelijke strategieën voor manipulatie van PRRs in een therapeutische setting aangewakkerd. Zo worden diverse TLR liganden op dit moment getest als adjuvantia in vaccins voor de behandeling van chronische virale infecties en kanker. De hoop is dat deze nieuwe klasse adjuvantia, met de potentie om de efficiëntie van talrijke onderdelen van de immuunrespons te verbeteren, de effectiviteit van deze vaccins kan verhogen om zodoende de veelal zwakke immuunrespons tegen bijvoorbeeld tumor-antigenen, die doorgaans weinig verschillen van zelf-antigenen, te versterken. Anderzijds zijn ongereguleerde PRR responsen geassocieerd met een scala aan immunologisch gemedieerde aandoeningen. Een zeer illustratief voorbeeld is de bevinding dat mutaties in de NLR NALP3 ten grondslag liggen aan een aantal zeldzame auto-inflammatoire ziekten, zoals *Muckle-Wells syndrome* en *familial cold autoinflammatory syndrome*. Dat fundamenteel onderzoek naar de functie van PRRs een belangrijke bijdrage kan leveren aan het ontrafelen van de pathofysiologie van dergelijke aandoeningen en het ontwerp van een rationele therapie wordt duidelijk wanneer men het succes van IL-1 receptor antagonisten bij de behandeling van bovengenoemde ziekten aanschouwt, nadat *in vitro* studies hadden aangetoond dat NALP3 een belangrijke rol speelt bij de productie van IL-1β. De centrale rol van PRRs in de regulatie van diverse inflammatoire mediatoren, maakt deze receptoren mogelijkerwijs nog aantrekkelijker als farmacotherapeutisch aangrijpingspunt. Eén potentiële strategie voor het ontwerp van PRR-gerichte therapieën is het in kaart brengen van de immuunsuppressieve mechanismen van pathogenen, met name virussen. Men kan zodoende gebruik maken van de evolutionair bepaalde optimalisatie van immunologisch actieve virale eiwitten als ware het een natuurlijk ‘drug development program’. Analyse van de exacte interacties tussen gastheer en pathogeen kan zo mogelijk bijdragen tot de identificatie van key drug targets en de weg openen naar nieuwe therapeutische mogelijkheden voor de behandeling van inflammatoire aandoeningen.

Dankwoord

Promoveren doe je niet alleen, zeker niet in het kleine dorp dat TIL heet, met meer dan 50 inwoners bij wie tips en trucs kunt vragen, spullen kunt lenen en altijd wel iemand in de buurt bij wie je even mag klagen als 'de pipetten alweer op zijn' ☺….Er zijn dus een heleboel mensen die ik graag wil bedanken voor hun directe of indirecte hulp bij het voltooien van dit proefschrift:

Graag begin ik met mijn promotor, Prof. Dr. Adema. Beste Gosse, ik kan me nog goed herinneren dat ik ten tijde van mijn sollicitatie eind 2002 best wel 'even' twijfelde tussen het NKI en TIL, maar heb al vaak gezegd geen dag spijt te hebben gehad van de keuze die toen is gemaakt. Voor een belangrijk deel is dit te danken aan de plezierige samenwerking die er tussen ons altijd is geweest. Ik heb de afgelopen jaren bij alle aspecten van mijn promotie-onderzoek veel profijt gehad van de kennis, creativiteit en ervaring die je met mij gedeeld hebt en daarvoor ben ik je erg dankbaar. Ondanks je drukke agenda was er altijd ruimte voor ons wekelijkse brainstorm uurtje en om even binnen te vallen als data 'echt niet kon wachten'. Ook van onze 'off-the-record praatjes' en de wijze lessen (van wijn tot serieuzer zaken) heb ik veel opgestoken. Bedankt voor vijf zeer aangename en leerzame jaren!

Dan Dr. van Kuppeveld, mijn co-promotor. Beste Frank, het was goed toeven op het grensvlak tussen DCs en picornavirussen en het is gebleken dat een combinatie van die twee tot spannende resultaten kan leiden! Wie weet komen we er zelfs nog achter of de DC dan toch de 'missing link' is tussen diabetes en enterovirussen. Bedankt voor alle goede ideeën en suggesties die je hebt ingebracht tijdens onze MMB/TIL meetings en de adviezen met betrekking tot het schrijven van goede artikelen. Het was fijn samenwerken!

Mijn tweede promotor, Prof. Dr. Galama. Beste Joep, bedankt dat je ondanks je drukke bezigheden in het ziekenhuis toch vaak de tijd hebt gevonden om bij onze MMB/TIL meetings aan te schuiven. Je enthousiaste reactie als Barbara en ik mooie data konden laten zien werkte altijd erg motiverend! Ook bedankt voor alles wat je me hebt bijgedragen over enterovirussen en je snelle respons bij allerhande vragen omtrent papers en het proefschrift!

Prof. Dr. Figdor. Beste Carl, bedankt voor de kans die je me hebt gegeven om op jouw lab promotie-onderzoek te kunnen doen. Het TIL, met zijn internationale karakter, het brede spectrum van onderzoeksprojecten (letterlijk 'from bench to bedside'), gecombineerd met de mooie successen die in de loop der jaren op het TIL zijn behaald (met als klapper je Spinozaprijs in 2006), werken voor een jonge onderzoeker erg motiverend! Ik hoop dat de tumorimmunologie nog veel profijt zal hebben van jouw creatieve ideeën.

De leden van de manuscript- en promotiecommissie: Prof. Dr. Schalkwijk, Prof. Dr. Berden, Dr. Roep, Prof. Dr. Figdor, Prof. Dr. Lambrecht, Prof. Dr. Netea, Dr. De Waal Malefyt en Prof. Dr. Wiertz wil ik graag bedanken voor de tijd en moeite die ze hebben geïnvesteerd in het doornemen van het proefschrift en de voorbereiding van de oppositie.

Dr. De Waal Malefyt. Beste Rene, de liefde voor DCs is bij DNAX begonnen. Ik kan me nog herinneren dat je op het witte bord voor het eerst uitlegde wat een Toll-like receptor was. Mijn stage in Palo Alto heeft me echt enthousiast gemaakt voor de immunologie en ik vind het ontzettend leuk dat je plaats wilt nemen in de promotiecommissie!

Barbara en Martijn: mijn paranimf duo!
Martijn: Goed dat je bent teruggekomen naar het TIL na je tijdelijke uitstapje naar Synthon (what a great time it was…). Eerlijk is eerlijk: de muizengroep (en het buren-Utje) waren niet

hetzelfde zonder 10-us! Met wie anders na 18.00 luisteren naar de 'betere' muziek die jouw MP3 lijst het lab te bieden heeft! Ook al hebben we nooit direct samengewerkt, toch waren jouw tips en trucs als 'ervaren AIO' in het eerste jaar meer dan welkom. Ook bedankt voor je hulp op allerlei andere vlakken; van APK's voor de laptop tot hulp bij de layout van mijn proefschrift en de keuze van de feestlocatie, 10-us weet raad! We gaan er een mooi feessie van maken!

Barbara: er was natuurlijk geen logischer keus dan jij! Met wie anders kan ik het zo goed hebben over Vero uptake, freeze-thaw cycles en ISG upregulation zonder gek aangekeken te worden? We hebben samen menig experimentje en flink wat uren in het kweeklab achter de rug en dat is altijd bijzonder gezellig geweest! Bovendien was het ook nog eens erg goed voor de conditie (hoe vaak zouden we tussen 1 en 5 heen en weer gerend zijn in al die jaren)? Bedankt voor de productieve en leuke samenwerking die we hebben gehad! Veel succes met de laatste loodjes van jouw onderzoek (ik hoop dat de eilandjes werken)! Ik weet zeker dat er een mooi boekje komt te liggen!

Liza. Met jou als technician was het laatste jaar van mijn promotie in alle opzichten 'dynamisch' te noemen (…). Dat de vele qPCRs, RNA isolaties en confocal kleuringen die je hebt gedaan tot mooie dingen hebben geleid blijkt wel uit het feit dat je naam boven drie van de papers in dit boekje staat. ¡Muchos gracias por tu asistencia!

Roger/Sjeetje! Een groot gemis voor het TIL dat jij bent weggegaan. Niet alleen omdat je de onbetwiste Treg koning bent, maar zeker ook omdat je een altijd goedgehumeurde en immer geduldige collega was! Ik heb veel geleerd van je (van Treg biology tot foute moppen). Ik weet zeker dat er mooie dingen binnen de dikke muren van SP gebeuren (al mag je ons die niet vertellen)…

Stefan: Bedankt voor al je hulp en advies en natuurlijk gewoon een hele leuke tijd (altijd fijn om met iemand op dezelfde golflengte te zitten)! Super dat jouw Amerikaanse avontuur met Femke door kon gaan! Ik hoop dat je daar mooie resultaten zult behalen (who needs nibbling DCs)! Ik verheug me enorm op onze trip naar San Diego!

Alle muizengroep leden: Alie, Annemiek, Inge, Ingrid, Johan, Jori, Marjolijn, Martijn, Stefan en Wendy, heel erg bedankt voor de altijd interessante DLMs en alle adviezen die ik in de loop der jaren van iedereen heb mogen ontvangen. Een bijzonder woord van dank voor de gezellige Utje 2 en 3 buren van de laatste periode: Inge, Johan, Jori, Marjolijn, Martijn, Stefan en Wendy (Inge B, je hebt je goed staande gehouden tussen al dat muizengeweld…), het was een mooie tijd! Jammer dat ik de laatste tijd zoveel weg van het lab was! Van iedereen heb ik wel wat geleerd en daarbij wil ik ook zeker de 'oude' muizengroep niet vergeten; Robbert, jouw creativiteit en nauwkeurigheid waren een goed voorbeeld voor een startende AIO; Frank W, ik zal nooit vergeten dat HO werkelijk OVERAL bij betrokken is ☺; Liz, tattooing didn't work out so well for you, but it was great fun being neighbors. Thanks for all the English lessons!

Bas, leuk om in de allerlaatste fase nog samen aan een paper te werken!

Alle andere Tillers, helaas is het met zo'n groot lab niet mogelijk iedereen persoonlijk te bedanken, ook al hebben jullie dat zeker verdiend! Iedereen bedankt voor de hulp bij FACS, confocal experimenten, het 'echte' moleculaire werk (ik weet nu in ieder geval dat Eco, Bam en Xho niet de nieuwste telefoonmodellen zijn), kweken, bestellingen en nog veel meer! En

natuurlijk Louise en Jeanette bedankt voor al jullie hulp en secretariële ondersteuning van de afgelopen jaren!

Maar…dat je met Tillers zoveel meer kunt doen dan alleen werken blijkt wel uit alle leuke dingen die we in de afgelopen jaren samen hebben ondernomen: ski-trips naar Frankrijk (Ben, Dani, Hans, Joost, Martijn, Stefan!), ongeëvenaarde X-mas parties, Belgische biertjes drinken na een mooi congres in Brugge (guys: what happens in Brugge, stays in Brugge), Dagjes Uit, Kroegentochten, Filmavonden, Poker-events, maar ook de gezellig lunchpauzes met een tafel vol Tillers (en gratis fruit)! De foto's op de H:schijf liegen niet: it was fun! Bedankt daarvoor!

Ook veel dank aan alle niet-Tillers die aan dit boekje hebben bijgedragen:

Prof. Dr. Netea. Beste Mihai, hartelijk bedankt voor al je input rondom onze NOD2 papers. Ik bewonder je veelzijdigheid en vind je een inspirerend voorbeeld van de wijze waarop fundamenteel onderzoek en patiëntenzorg gecombineerd kan worden!

Beste Gerben: Het was leuk brainstormen met je over NOD2 en aanverwante zaken. Bedankt voor de samenwerking tijdens het maken van ons artikel! Leuk om je nu ook in de kliniek tegen te komen! Ik zie uit naar het lezen van jouw proefschrift!

Beste Dirk: bedankt voor je altijd snelle reply bij de vragen die ik had rondom NOD2 en Crohn!

Mike, bedankt voor de eerste stappen samen binnen het 'virus-DC project'.

Beste CHL'ers, vooral Louis, Frans en Rob: bedankt voor jullie hulp bij alle PCR experimenten en het opzetten vd EBV-cellijnen.

Beste Marijke en Mieke, ook jullie bedankt voor jullie hulp bij de PCR experimenten en primer perikelen.

Beste Huib, bedankt voor alle hulp rondom de confocal experimenten.

Loutje! Bedankt voor het maken van een prachtige cover!

Naast al dat pipetteren is het natuurlijk erg belangrijk om ook af en toe eens te ontspannen en met wie kon dat beter dan met jullie:

Sanne, blue Dory-fish! What can I say other than *BEEEEHH*...thanks for just being Dory and a lot of special moments! Het is me, ondanks verwoede pogingen, nooit gelukt je enthousiast te krijgen over 'de celletjes' (gelukkig duurt de promotie maar een uurtje). Super dat je een echte ER doc wordt, it's the perfect job for you! Ik hoop dat we samen nog vele cupjes tea zullen drinken!

Bart, Chantal, Erika en Niek, samen met Sanne zijn wij samen in Nijmegen (bijna) vanaf het eerste uur! Echt geweldig dat wij na al die jaren nog zoveel contact hebben! Omdat we elkaar al zolang kennen, zijn, beschouw ik jullie als mijn Nijmeegse surrogaat-familie ☺ (B/C, wanneer komen jullie nu in dat mooie torentje hier tegenover wonen)? We hebben al veel meegemaakt: samen op de uni, afstuderen, onze eerste 'echte' banen en natuurlijk een bruiloft! Ook al hebben we het allemaal heel druk, super dat we deze dag samen kunnen vieren!

Stefan (GLF!), Janine, Peter en Celine: Wat een geweldig leuke weekendjes, etentjes en vakanties hebben wij met zijn allen al gehad! Het maakt niet uit waar we zijn: van het warme Frankrijk (1000 potjes kaarten en kilo's blauwe kaas) tot het koude Zwitserland (ieder jaar een beetje sneller de berg af) of gewoon hier in Nederland. Ik ben blij dat ik jullie heb leren kennen, natuurlijk samen met….Guus, Karlien, Koen en Anke: jammer dat wij elkaar minder vaak zien dan we zouden willen (1 x per 6 weken SVB weekendje is net iets te ambitieus), maar met co-schappen in Doetinchem en Anouk nog even in Rotterdam zullen we dat het komende jaar eens gaan verbeteren!

Bertjan, Mirjam, Martijn. Bedankt voor de talloze stapavonden (en ochtenden), de mooie volleybal/kleedkamer/toernooi herinneringen en natuurlijk een onvergetelijke trip in de VS (zal de neerstortende meteoriet en de 'beertjes' aan de tent nooit vergeten)!

De squashers: Luuk, Martijn, Hugo. Hopelijk houden we ons wekelijkse 1.5 uurtje zweten nog lang vol!

Maar natuurlijk begint het allemaal met 'thuis': Patrick, BB! We hebben samen menig paar schoenen versleten tijdens het voetballen op 'het garagepad'. Later verruilden we de voetbal voor de volleybal en dat beviel nog beter: zes superleuke jaren in één team met dito avonden in Sev en de Oortjes (ieder weekend was 't weer mooier)! Ook al zien we elkaar nu wat minder vaak, het blijft altijd gezellig als ik weer in Middelburg ben. Ik wens jou veel geluk samen met Heidi!

Lieve pa en ma: jullie zijn het volmaakte voorbeeld van hoe een lang en gelukkig huwelijk eruit ziet en hebben altijd gezorgd voor de perfecte thuisbasis voor Pat en mij! Bedankt voor jullie onvoorwaardelijke steun en interesse en dat jullie altijd achter de keuzes hebben gestaan die wij genomen hebben. Ik hoop dat we samen nog vele mooie momenten mee zullen maken, dit is er zeker één van!

En tenslotte Anouk! Mijn lieve krullenbol, bedankt voor de geweldige tijd die we tot nu toe samen gehad hebben! Ook al is het soms niet leuk om zo ver uit elkaar te wonen, het maakt de weekends samen in Rotterdam, Gent en Nijmegen alleen maar des te fijner…Ik ben trots op je dat je op al die plekken zo gemakkelijk je draai vindt en natuurlijk dat je promotieonderzoek zo goed gaat! We hebben toch wel veel te danken aan die gekke DCs... Ik heb zin om samen met jou Leiden te gaan verkennen (of Den Haag, of waar dan ook). Enne, dat kookeiland komt er wel, misschien zelfs een Gijsje…!

Curriculum Vitae & Publication List

Curriculum Vitae (Nederlands)

Matthijs Kramer werd geboren op 10 april 1979 te Middelburg. Na afleggen van het eindexamen VWO aan de Stedelijke Scholengemeenschap Middelburg (SSGM) startte hij in 1998 met de opleiding Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen (thans Radboud Universiteit Nijmegen) met als hoofdvakken Toxicologie en Pathobiologie. Tijdens de studie werd 6 maanden stage gelopen bij de afdeling Toxicology and Drug Disposition van Organon (Schaijk, Nederland) onder begeleiding van Dr. Diels van den Dobbelsteen, waar onderzoek werd gedaan naar de toepasbaarheid van de 'Micromass teratogeniteitstest' voor het screenen van nieuwe farmacologische compounds. Een tweede stageperiode van 7 maanden werd voltooid bij DNAX Research Institute (Palo Alto, Californië, VS) onder supervisie van Dr. R. de Waal-Malefyt. Het onderzoek betrof de ontwikkeling van een test waarmee de productie van het toen pas ontdekte cytokine IL-23 door monocyten en dendritische cellen werd bestudeerd. Na afleggen van het doctoraal examen in december 2002 is hij van januari 2003 tot december 2007 aangesteld geweest als Junior Onderzoeker op de afdeling Tumor Immunologie. Onder begeleiding van Prof. Dr. G.J. Adema werd het promotieonderzoek uitgevoerd zoals beschreven in dit proefschrift. Tijdens het onderzoek heeft hij de mogelijkheid gekregen om de theoretische component van de studie Geneeskunde te voltooien en in februari 2008 is gestart met de afronding van de klinische fase (co-schappen).

Curriculum Vitae (English)

Matthijs Kramer was born on April 10, 1979 in the city of Middelburg in The Netherlands. After graduating from pre-university education at the Stedelijke Scholengemeenschap Middelburg (SSGM), he began the Master study Biomedical Sciences at the Catholic University Nijmegen (currently Radboud University Nijmegen). During his studies, he performed a six month traineeship at Organon within the Department of Toxicology and Drug Disposition (Schaijk, The Netherlands). Under the guidance of Dr. D.J. van den Dobbelsteen, he focused on the applicability of the Micromass teratogenicity test for toxicological screening of novel pharmacological compounds. A seven month traineeship under supervision of Dr R. de Waal Malefyt was completed within DNAX Research Institute (Palo Alto, CA, USA), where a test was developed to study the production of the newly discovered cytokine IL-23 by monocytes and dendritic cells. After graduating from university in December 2002, he worked as a Junior Researcher at the Department of Tumor Immunology from January 2003 until December 2007. Here, the studies described in this thesis were performed under the supervision of Prof. Dr. G.J. Adema. During this period, he was also given the opportunity to finish the theoretical component of his medical training. His medical internships have started in February 2008.

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